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STUDIES ON CYTO-TAXONOMY AND REPRODUCTION IN THE ENCHYTRAEIDAE

WITH NOTES ON PARTHENOGENESIS AND POLYPLOIDY IN THE ANIMAL KINGDOM[1]

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I. INTRODUCTION

In previous publications from the Mols Laboratory certain biological (NIELSEN, 1952—53, 1954, 1955 a, b, c, d; CHRISTENSEN, 1956; CHRISTENSEN and O'CONNOR, 1958); taxonomical (NIELSEN and CHRISTENSEN, 1959 and 1961) and cytological (CHRISTENSEN, 1960; CHRISTENSEN and NIELSEN, 1955), problems in the Enchytraeidae have been dealt with. The present paper is another contribution to this series.

The only previous observations on the cytology of Enchytraeidae are reported by VEJDOVSKY in his classical paper, *Neue Untersuchungen über die Reifung und Befruchtung* (1907), where he quotes chromosome numbers of five species, together with some observations on bivalent formation and other details about the first meiotic division. That only one paper on the cytology of Enchytraeidae has been published in the past is certainly due to the taxonomical difficulties encountered in this little known family of Oligochaeta. In the present paper the taxonomy follows NIELSEN and CHRISTENSEN (1959 and 1961).

The observations are presented in the following way: In *section 1* the chromosome numbers are summarized in *Table 1*, the morphology of somatic chromosomes is briefly described and the occurrence of polyploidy mentioned. In *section* 2 the different reproductive mechanisms are described. *Section* 3 contains descriptions of different bivalent types. Within each type the cytology of amphimictic and partheno-

[1] Joint publication from Molslaboratoriet, Femmøller, Institute of Genetics and Zoological Laboratory, University of Copenhagen.

genetic forms is described, and when both diploid and polyploid forms exist these are again considered separately. Finally in *section 4* variations in the diakinetic contraction of the bivalents is briefly mentioned, and the anaphasic disjunction of the homologues, in some species where the egg nucleus is in first meiotic anaphase (AI) when the eggs are laid, is briefly described.

II. MATERIAL AND METHODS

The Enchytraeidae is a family of Oligochaetes like the earthworms, Lumbricidae, the Tubificidae and the Naididae, just to mention the most prominent European families within the order. Enchytraeid worms are found in littoral and terrestrial habitats, they are especially abundant in humid habitats where organic material accumulates, i.e. in wrack beds, moorland, bogs and compost heaps.

The Enchytraeidae are hermaphrodites, with male and female organs in the same individual. The *male reproductive system* includes 1) a pair of small *testes* in segment xi, 2) a pair of *sperm funnels* in the same segment connecting to 3) the *vas deferens* that leads to 4) the *male pores* opening ventrally in segment xii. The early spermatogonial divisions take place in the testes proper, but the maturing male cells soon separate from the testes and float freely in the body cavity of segment xi or enter a special organ — the *seminal vesicle*. The multiplying spermatogonial cell forms a rounded body which at the first meiotic metaphase (*MI*) usually contains 64 cells with complete synchronization.

The mature spermatozoa are discharged through the *vas deferens* during copulation. The *female reproductive system* includes 1) a pair of *ovaries* anteriorly in segment xii and 2) a pair of *female openings* posteriorly in the same segment immediately behind the male pores. The female system also includes 3) a pair of *spermathecae* (*seminal receptacles*) located in segment v and opening to the exterior ventrally in the intersegmentary groove iv/v. In the spermathecae the spermatozoa received during copulation are stored until required for fertilization of the eggs in the cocoon.

The maturing female cells usually leave the ovary as small round bodies consisting of appr. 16 cells each and floats freely in the cavity of segments 13 and 14. One of these cells commences growing and develops into the future egg, while the remaining cells degenerate. In the growing oocyte the bivalents are usually Feulgen-negative, but when growth is nearly completed they become stainable; they are now in the

diplotene stage and soon proceeds into diakinesis. Finally the nuclear membrane breaks down and the bivalents are arranged in the first meiotic metaphase (MI). In the majority of Enchytraeid worms the eggs remain in this stage until they leave the worm (rare exceptions to this rule are described on p. 426). The duration of this stage depends on environmental conditions; at low temperatures, when the production of cocoons is at a minimum, it may last for several months.

For the isolation of Enchytraeidae from soil samples NIELSEN's (1952—53) and O'CONNOR's (1955) techniques have been used.

For studying the restoration of the somatic chromosome numbers in newly laid eggs it is necessary to keep the worms in special breeding chambers which allow direct observation under the dissecting microscope because the cocoons, in which the eggs are laid, are of a small size, and in addition most species cover up their cocoons with sand grains and plant material from the substratum in which they live. Worms from habitats with much organic matter are kept in breeding chambers described by CHRISTENSEN (1956). For worms living in mineral soil the following technique has been used with some success: A thin layer of the material used as substratum is placed on the bottom of a small petri-dish, larger sand grains and other hard particles must be carefully removed. As many cover slips as possible are placed on the substratum, broken bits being used at the perifery. When worms are introduced they will soon crawl into the substratum between the cover slips and the bottom of the petri-dish. Finally the lid is placed on the petri-dish and the culture is ready for use; it can conveniently be inspected under the dissecting microscope from above — by removing the lid — as well as from below — by inverting the petri-dish. A satisfactory moisture status is maintained in the substratum by adding a few drops of water daily.

The cytological techniques used have been described earlier (CHRISTENSEN and NIELSEN, 1955; CHRISTENSEN, 1960). Here only a modification of the orcein-squash technique for making preparations of the oocyte MI is described. The technique has proved useful in the daily routine work and it offers the advantage that fixed material may be kept for some time: 1) the worms are fixed *in toto* in Carnoy's fluid (abs. alcohol:acetic acid, 3:1) for c. one hour; 2) 96 % alcohol for c. 10 minutes; 3) 96 % alcohol over-night; 4) back to Carnoy's fluid for c. 15 minutes; 5) 45 % acetic-orcein in a watch-glass. In the acetic acid the yolk-rich eggs swell and eventually burst the body wall. The process is followed in transmitted light under the dissecting microscope, and

when bursting approaches the worm is 6) transferred to a drop of acetic-orcein on a slide. The egg is dissected out and the remains of the worm removed. The material may be kept in the second change of 96 % alcohol at least for a fortnight without detrimental effects.

III. OBSERVATIONS

Section 1. Chromosome numbers

Descriptions of the species in the following list are given in NIELSEN and CHRISTENSEN (1959 and 1961).

VEJDOVSKY (1907) reports chromosome numbers of the following five species: *Mesenchytraeus flavus* n=16, *M. armatus* (*setosus*) n=16, *Fridericia hegemon* n=32, *Enchytraeus humicultor* n=16 and *E. adriaticus* n=12. VEJDOVSKY's observations have been confirmed and the three first named species are included in Table 1. The two latter species, however, are insufficiently described and identification is not possible, there are even doubts as to their generic position.

In an earlier report chromosome numbers from 12 species were reported (CHRISTENSEN and NIELSEN, 1955). These counts were based on spermatocyte divisions and the present investigation necessitates a few corrections. In *Lumbricillus helgolandicus* the number was reported as n=14, the correct number is n=13 (the n=14 counts were probably based on semi-adult individuals of *L. pagenstecheri*). The tetraploid cytotype n=34 of *Henlea ventriculosa* in the 1955 paper has later been described as a separate species *H. jutlandica* (NIELSEN and CHRISTENSEN, 1959). The 2n=120—140 cytotype of *H. ventriculosa* and n=32 cytotype of *Fridericia ratzeli* have not been refound. In the same paper 2n=180—200 was reported from *Cognettia sphagnetorum*. Since worms with mature eggs have not been found in the original habitat, it has not been possible to check the number, hence it is omitted from Table 1.

In CHRISTENSEN and NIELSEN (1959), the present author has contributed some preliminary chromosome data. Since then more material has been available for several of the species, and this has given a few corrections: *Cernosvitoviella atrata* n=13, *Henlea cf. heleotropha* n=16, *Buchholzia appendiculata* n=18 and *Marionina spicula* n=17. The n=64 cytotype of *Fridericia ratzeli* is omitted because it has not been confirmed due to lack of material.

It will appear from Table 1 that in several cases more than one chromosome number is reported from the same morphological species, usually in association with intraspecific polyploidy. In such cases different *cytotypes* are distinguished. A genetical species concept, cf. DOBZHANSKY (1951, p. 262), requires that every such cytotype should be established as a separate species, since it is genetically isolated from other cytotypes within the same "species". However, in most cases cytotypes within the same species cannot be distinguished unless cytological criteria are applied, and from a practical point of view it is, therefore, reasonable to retain these collective species, but, of course, to state expressly where cytological heterogeneity is at hand. In a few cases there is an indication of intra-specific chromosomal variation including only a few chromosomes. These cases need further investigation, and are not considered

TABLE 1. *Chromosome numbers of Enchytraeidae, their meiotic type, degree of ploidy, and reproduction.*

Species	Obs. no. of chromosomes		Chiasmatic meiosis		Achiasmatic meiosis		Reproduction	Fig.
	biv.	univ.	dipl.	polypl.	dipl.	polypl.		
Mesenchytraeus								
glandulosus	16	32	+				parth.	33, 34, 62, 63
pelicensis	32	64		+			„	35, 64, 65, 130, 131
armatus	16[1]	32	+				amph.	128, 129
flavus	16[1]	32	+				„	
beumeri	16		+				„	
Cernosvitoviella								
atrata	13	26	+				„	1, 49
Cognettia								
glandulosa	c. 54	c. 108		+			asex. parth.	29—32, 99
sphagnetorum	c. 54			+			asex.	
	c. 160			+			„	
Lumbricillus								
arenarius	17	c. 34	+				amph.	92
bülowi	15	30	+				„	5, 95, 96
knöllneri	c. 15		+				„	
tuba	13	26	+				„	8, 76
helgolandicus	13	26	+				„	72—75
kaloensis	13	26	+				„	77
rivalis	13	26	+				„	6, 57
lineatus	13	26	+				„	
		39[2]					parth.	
viridis	14	28	+				amph.	2, 48
pagenstecheri	14	28	+				„	91
Enchytraeus								
minutus	16	32	+				amph.	98
buchholzi	18	36	+				„	78—82
	36	72		+			„	83—86
lacteus	18	36	+				„	87
	36	c. 72		+			„	88
	c. 54			+			„ ?	
	72	c. 144		+			„	89, 90
coronatus	18	36	+				„	66—71
	c. 36			+			„ ?	
albidus	21	42	+				„	93
capitatus	21	42	+				„	18, 53
irregularis	21		+				„	94
Hemienchytraeus								
bifurcatus	16	32	+				amph.	12, 52

TABLE 1 (continued).

Species	Obs. no. of chromosomes		Chiasmatic meiosis		Achiasmatic meiosis		Reproduction	Fig.
	biv.	univ.	dipl.	polypl.	dipl.	polypl.		
Achaeta								
camerani	c. 18		+				amph.	
parva	12		+				„	54
bohemica	c. 27			?			„	45
danica	32			?			„	55
affinis	36			?			parth.?[3]	50
bulbosa	150—160					+	?	120
Enchytronia								
parva	24	c. 48			+		amph.	110
Fridericia								
bulbosa	32					+	amph.	22
bulboides	32					+	„	26
callosa	c. 32					+	„	
striata	32					+	„	25
paroniana	c. 32					+	„	
bisetosa	(30)32[4]	64				+	„	24
	c. 56	c. 112				+	parth.?[5]	115
maculata	26	52				+	amph.	17, 23
	45	90				+	parth.?[3]	16, 117
leydigi	32					+	amph.	19
	60	c. 120				+	?[5]	27
connata	32					+	amph.	
	56	c. 112				+	parth.?[5]	116
	64	c. 128				+	amph.	28
perrieri	32					+	„	
galba	32	64				+	„	21
ratzeli	40	80				+	parth.	111, 119
	56	112				+	„	35, 37, 118
	80—90					+	?	
gracilis	32	c. 64				+	amph.	20
hegemon	32[1]	64				+	„	
Buchholzia								
appendiculata	18	36			+		asex. amph.?	13, 109
fallax	18(19)[4]	36—			+		amph.	100—105
	36—(38)[4]	72—				+	„	106—108
Hemifridericia								
parva	28					+	amph.	
	c. 42					+	„	
Henlea								
cf. heleotropha	16	32	+				amph.	3, 39

TABLE 1 (continued).

Species	Obs. no. of chromosomes		Chiasmatic meiosis		Achiasmatic meiosis		Reproduction	Fig.
	biv.	nniv.	dipl.	polypl.	dipl.	polypl.		
perpusilla	17	34			+		amph.	9, 113, 126, 127
	c. 34[6]	c. 68				+	„	114
nasuta	17	c. 34	+				„	41
similis	17		+				„	40
ventriculosa	17	34	+				„	38
jutlandica	c. 34			+			„	59, 60
Bryodrilus								
ehlersi	15	30	+				amph.	15, 58
Marionina								
subterranea	18	36			+		amph.	121—124
sjaelandica	c. 18	c. 36			+		„	14, 125
southerni	18	c. 36	+				„	11, 46
argentea	18	36	+				„	42
spicula	17	c. 34	+				„	47
communis	15	30	+				„	10, 56
tubifera	c. 24		+				„	43
vesiculata	c. 20		+				„	44
clavata	c. 38			+			„	61
libra	17		+				„	
riparia	15(17)[4]		+				„	51
appendiculata	17		+				„	97
Generis incerti (*Enchytraeus*)								
humicultor	16[1]	32	+				amph.	
adriaticus	12[1]	24	+				„	
71 Total number of species	88 Total number of cytotypes		41	14	6	26	Number of di- and polyploid cytotypes with *chiasmatic* and *achiasmatic* bivalents respectively	

[1] see also VEJDOVSKY (1907); [2] asynaptic meiosis, see CHRISTENSEN (1960); [3] athecate form; [4] probably heterogenous, the chromosome number varies between different populations; [5] sperm production reduced compared with the n=32 cytotype; [6] apparently two forms (cf. p. 394).

separately here, cf. Table 1, foot-note 4. Similarly the tetraploid cytotype of *Henlea perpusilla* apparently comprises two forms, in one of these the egg nucleus remains in the first meiotic metaphase (MI) until the egg is laid, in another, the first meiotic metaphase is of short duration and the nucleus proceeds into first meiotic anaphase (AI) before the egg is laid.

In Table 1 the worms are divided into two main groups according to bivalent behaviour during meiosis: 1) *chiasmatic meiosis* (cf. p. 403) and 2) *achiasmatic meiosis* (cf. p. 415). A third category: 3) *asynaptic meiosis*, to which only *Lumbricillus lineatus 3x* belongs is not included in the table, cf. CHRISTENSEN (1960) and p. 425. The two bivalent types are again divided into diploids and polyploids. In column 7 the mode of reproduction is given: amphimictic (amph.) cf. p. 399, parthenogenetic (parth.) cf. p. 399, and asexual (asex.) cf. p. 402.

Mitoses are obtained from spermatogonial and in a few cases from blastomere divisions. Due to the small size of the chromosomes in spermatogonial divisions, is it often difficult to determine the position of centromeres, hence the exact morphology of a complete somatic set is only known for a small number of species. Metacentric as well as acrocentric chromosomes are present, and, as a general rule, the larger chromosomes are metacentric and the smaller ones acrocentric (cf. Figs. 1—18). In the genus *Fridericia*, the chromosomes of spermatogonial mitoses are very small, but the special type of meiosis, the *achiasmatic* meiosis, characteristic of this and a few other genera (cf. p. 415) shows in the early first meiotic metaphase stage an opening out in the centromere region of the parallely paired homologues, and the morphology of the chromosomes is easily seen at this stage. Figs. 19—28 show oocyte MI plates of some *Fridericia* species.

It will appear from Table 1 and the histogram in Fig. 132 that haploid chromosome numbers range from 12 to 150—160, with two peaks, viz. one between 12 and 21 and another at even numbers from 32 to 36. The variation in chromosome numbers within the family is due to inter-, as well as, intra-generic variation, in a few genera all species have the same chromosome number, while in others there is wide variation.

Figs. 1—3 and 5—17, spermatogonial mitoses, ×1500; Figs. 4 and 18, blastomere mitoses, ×1000. — Fig. 1: *Cernosvitoviella atrata*; 2: *Lumbricillus viridis*; 3: *Henlea cf. heleotropha*; 4: *Mesenchytraeus armatus*; 5: *Lumbricillus bülowi*; 6: *L. rivalis*; 7: *Enchytraeus minutus*; 8: *Lumbricillus tuba*; 9: *Henlea perpusilla*; 10: *Marionina communis*; 11: *Marionina southerni*; 12: *Hemienchytraeus bifurcatus*; 13: *Buchholzia appendiculata*; 14: *Marionina sjaelandica*; 15: *Bryodrilus ehlersi*; 16: *Fridericia maculata* n=45; 17: *F. maculata* n=26; 18: *Enchytraeus capitatus*.

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It appears from Table 1 that several cases of polyploidy occur among the Enchytraeidae. In some cases the chromosome number of a given species is a multiple of the number characteristic of related species: *Mesenchytraeus pelicensis* n=32 (related to *M. glandulosus* n=16), *Henlea jutlandica* n=c. 34 (related to *H. ventriculosa* n=17), *Marionina clavata* n=c. 38 (related to *M. vesiculata* n=c. 20). In other cases chromosome numbers are known which are multiples of another number within the same morphological species (intra-specific polyploidy): *Cognettia sphagnetorum* n=c. 160, *Lumbricillus lineatus* 2n=39, *Enchytraeus buchholzi* n=36, *E. lacteus* n=36, c. 54 and 72, *E. coronatus* n=c. 36, *Fridericia connata* n=64, *Hemifridericia parva* n=c. 42, *Buchholzia fallax* n=36—(38), *Henlea perpusilla* n=c. 34.

In the examples just mentioned the presence of polyploidy is obvious. In other cases, however, the situation is more complex, since no basic complement is known of which the chromosome numbers in question are multiples:

1) Within the genus *Cognettia* the haploid numbers c. 54 and c. 160 are known, n=c. 160 is probably hexaploid with respect to n=c. 54, however, the true degree of polyploidy is certainly higher, since n=c. 54 is unlikely to be the basic haploid set for the genus; compared with chromosome numbers of all other genera, it is probably in itself a polyploid number.

2) Within the genus *Achaeta* the haploid numbers 12, c. 18, c. 27, 32, 36 and 150—160 are known. n=12 and c. 18 are probably original haploid numbers, whereas the remaining numbers may be of a polyploid nature, but the exact degree of polyploidy is not known.

3) *Hemifridericia parva* comprises two cytotypes, n=28 and c. 42, these are both multiples of 14, and 28 is probably a tetraploid and 42 a hexaploid number.

4) Chromosome numbers are reported from 14 different *Fridericia* species, comprising 21 different cytotypes, n=32 is by far the commonest haploid number, 12 among 21 cytotypes, and it is probably the ancestral number for the genus, from which all other chromosome numbers have been derived. The fact that the majority of the other chromosome numbers are known from cytotypes within species where also a 32-type occur supports this view. Compared with the other chromosome numbers of the family, it is seen that n=32 is on a level with some evident tetraploids (*Mesenchytraeus pelicensis*, *Henlea perpusilla* 4x, *H. jutlandica*). This suggests that the *Fridericia* species in question — compared with the general level within the family — are actually

Figs. 19—28, oocyte MI from some *Fridericia* species, ×1000. — Fig. 19: *leydigi* n=32; 20: *gracilis*; 21: *galba*; 22: *bulbosa*; 23: *maculata* n=26; 24: *bisetosa* n=32; 25: *striata*; 26: *bulboides*; 27: *leydigi* n=60; 28: *connata* n=64.

tetraploids. If this holds true the ancestral basic number is n=16, and the evolution of the genus has taken place at a tetraploid level. The following species and cytotypes are then tetraploids: *F. bulboides*, *bulbosa*, *callosa*, *striata*, *paroniana*, *bisetosa* n=32, *leydigi* n=32, *connata* n=32, *perrieri*, *galba*, *gracilis* and *hegemon*. *F. maculata* n=26 may then be hypo-tetraploid. Six haploid numbers higher than 32 are known, among these the somatic number of *F. connata* n=64 is an even multiple of a basic number of 16 (octoploid). The remaining numbers 40, 45, 56, 60 and 80—90 are undoubtedly of a polyploid nature, but the exact degree is not known.

It will then appear from Table 1 that chromosome numbers are reported from 71 different species comprising 88 different cytotypes, and no less than 41 cytotypes, or 47 %, are polyploids, ranging from triploidy to at least two octoploids.

The various polyploid cytotypes occur in the following numbers:

Triploids: 1 cytotype (*Lumbricillus lineatus 3x*).

Tetraploids: 22 cytotypes, although some of these may be either hypo- or hypertetraploids (*Mesenchytraeus pelicensis*, *Enchytraeus buchholzi* n=36, *E. lacteus* n=36, *E. coronatus* n=36, *Fridericia bulbosa*, *F. bulboides*, *F. callosa*, *F. striata*, *F. paroniana*, *F. bisetosa* n=32, *F. maculata* n=26, *F. leydigi* n=32, *F. connata* n=32, *F. perrieri*, *F. galba*, *F. gracilis*, *F. hegemon*, *Hemifridericia parva* n=28, *Buchholzia fallax* n=36—(38), *Henlea perpusilla* n=c. 34, *H. jutlandica*, *Marionina clavata*).

Hexaploids: 2 cytotypes (*Enchytraeus lacteus* n=c. 54, *Hemifridericia parva* n=c. 42).

Octoploids: 2 cytotypes (*Enchytraeus lacteus* n=72, *Fridericia connata* n=64).

In 14 polyploid cytotypes, the exact multiple of a basic haploid set is not known (*Cognettia sphagnetorum* n=c. 54 and c. 160, *C. glandulosa*, *Achaeta bohemica*, *A. danica*, *A. affinis*, *A. bulbosa*, *Fridericia connata* n=56, *F. bisetosa* n=56, *F. maculata* n=45, *F. ratzeli* n=40, 56 and 80—90, *F. leydigi* n=60).

Concerning the occurrence of polyploidy within the different bivalent types, cf. Table 1. Other aspects of this topic are discussed on p. 438.

Section 2. Reproductive mechanisms

Among the Enchytraeidae three kinds of reproduction have been found: 1) Amphimixis, 2) Parthenogenesis (associated with pseudofer-

tilization) and 3) Asexual reproduction (either obligatory or coexisting with sexual reproduction).

The study of reproductive mechanisms in Enchytraeidae presents several difficulties, it has, therefore, only been possible to analyze in detail a small number of species. The main difficulties encountered are: 1) like other Oligochaetes, the Enchytraeidae are all typical hermaphrodites, and although most species certainly crossbreed as a rule, there is no anatomical barrier to self-fertilization, and only breeding experiments can show whether or not cross-breeding is obligatory, 2) among the parthenogenetic Enchytraeidae hitherto examined only *Lumbricillus lineatus 3x* and perhaps *Fridericia maculata* n=45 produce no spermatozoa, these are therefore the only cases where parthenogenetic reproduction is certain *a priori*. In all other Enchytraeid worms cytological investigation is necessary in order to distinguish parthenogenesis from self-fertilization — or cross-breeding, 3) in all Enchytraeidae the restoration of the somatic chromosome number takes place after egg deposition, and the eggs are laid in cocoons which are very difficult to find in their natural habitats, hence it is necessary to keep the worms in special breeding chambers, see p. 389 and CHRISTENSEN (1956).

A. Amphimixis

Due to the technical difficulties already mentioned, the actual fusion of male and female pronuclei has only been observed in a small number of the species listed as amphimictic in Table 1. These are: *Mesenchytraeus flavus*, *M. armatus*, *Lumbricillus lineatus 2x*, *L. rivalis*, *L. pagenstecheri*, *Enchytraeus albidus*, *E. capitatus*, *Henlea ventriculosa*, *Fridericia galba*, *F. gracilis*. The extrusion of polar bodies and fusion of pronuclei follows the pattern described for *Lumbricillus lineatus 2x* by CHRISTENSEN (1960).

Other species are listed as amphimictic when all individuals meet the following requirements: 1) oogenesis and spermatogenesis completely normal, 2) spermatozoa always present in the spermathecae of sexually mature individuals.

B. Parthenogenesis

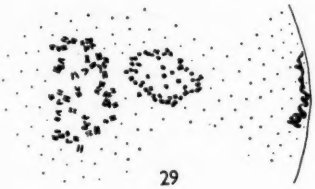
Parthenogenetic reproduction has been established in the following species: *Mesenchytraeus glandulosus*, *M. pelicensis*, *Lumbricillus lineatus 3x*, *Cognettia glandulosa*, *Fridericia ratzeli* n=40 and n=56. Less conclusive cytological evidence is available for *F. connata* n=56 and *F. bisetosa* n=56.

In all cases except *Lumbricillus lineatus 3x* (see below), the haploid number of bivalents is formed at the first meiotic division, and the first polar body is extruded (Figs. 29, 34 and 35). The chromosomes of the secondary oocyte are arranged in a second meiotic metaphase plane, but spindle formation is incomplete and no second anaphase occurs. The daughter chromosomes are kept together in the centromere region for a while, but eventually they separate (Figs. 29, 33 and 35) uncoil and lose their stainability (Fig. 34). All the chromosomes become included in a single nucleus with the somatic chromosome number which proceeds into interphase. Although the second meiotic division is not completed, the restoration of the somatic chromosome number (in these cases) is genetically and cytologically equivalent to that of fusion between the second polar body and the female pronucleus.

Apart from the cases where parthenogenesis has been verified cytologically, parthenogenetic reproduction is also likely to occur in a few other species. In *Achaeta affinis* and *Fridericia maculata* n=45 spermathecae are entirely absent, hence the worms must reproduce either by self-fertilization or by parthenogenesis. In *Fridericia leydigi* n=60 the production of spermatozoa is reduced compared with the n=32 cytotype, and in *Achaeta bulbosa* the high number of chromosomes may suggest parthenogenetic reproduction.

In *Cognettia glandulosa* and *Fridericia ratzeli* a smaller proportion of the developing embryos have only half the somatic chromosome number (Figs. 32 and 36). In these cases both polar bodies are extruded and the female pronucleus develops directly into the somatic nucleus. These embryos seem to develop completely normally, but it is unknown whether they hatch. Since the complete somatic chromosome set was found in the germ-line of all the individuals investigated, the "haploid" embryos probably never reach sexual maturity. The possible rôle of

Fig. 29, *Cognettia glandulosa*, newly laid egg, abortive MII (left), 1st polar body (in centre) and sperm head (right), semi-schematically, ×510. — Fig. 30, *C. glandulosa*, interphase (left), 1st polar body (right, on top) and male pronucleus (right, below), semi-schematically, ×510. — Fig. 31, *C. glandulosa*, early blastomere mitosis, appr. 108 univalents (left), 1st polar body (right on top) and male pronucleus (right, below), semi-schematically, ×510. — Fig. 32, *C. glandulosa*, early blastomere anaphase, appr. 54 univalents (left) and 1st and 2nd polar body (right), semi-schematically, ×510. — Fig. 33, *Mesenchytraeus glandulosus*, abortive MII from egg, ×670. — Fig. 34, *M. glandulosus*, uncoiling chromosomes in restitution nucleus (left) and 1st polar body (right), semi-schematically, ×510. — Fig. 35, *M. pelicensis*, abortive MII (left) and 1st polar body (right), semi-schematically, ×510. — Fig. 36, *Fridericia ratzeli* n=56, blastomere mitosis showing 56 univalents, ×510. — Fig. 37, *F. ratzeli* n=56, blastomere mitosis showing 112 univalents, ×510.



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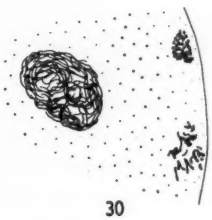

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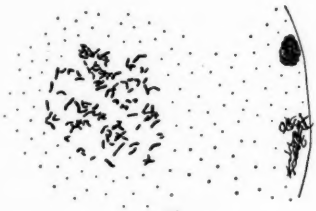

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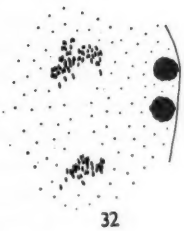

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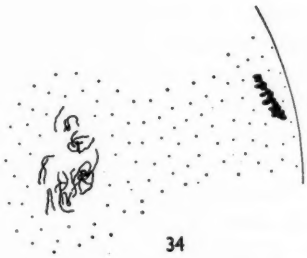

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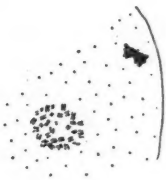

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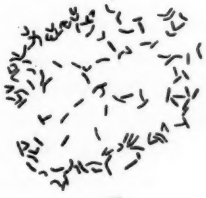

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the spermatozoa in the formation of these haploid embryos is dealt with below.

The special mechanism for the restoration of the somatic chromosome number in *Lumbricillus lineatus 3x* is described in CHRISTENSEN (1960). In this type spermatogenesis does not proceed beyond the spermatid stage and no mature spermatozoa are produced. In spite of this, the spermathecae of the triploid worms always contain spermatozoa. Experiments have shown, that these spermatozoa are provided by the diploid amphimictic cytotype of *L. lineatus*, and the experiments also proved that activation (pseudo-fertilization) by spermatozoa from the diploid cytotype is necessary for the successful development of the eggs of the triploid parthenogenetic cytotype (CHRISTENSEN and O'CONNOR, 1958).

Although *Cognettia glandulosa* reproduces parthenogenetically, spermatozoa are nearly always present in the spermathecae. In most cocoons spermatozoa occur, and one of these penetrates the egg membrane. The sperm-tail is lost (Fig. 29), the head begins to swell and the chromosomes become visible as thin, faintly stainable threads (Fig. 30). At this time the sperm-head is located close to the first polar body now extruded, both nuclei lie immediately below the egg membrane. In the polar body the chromosomes clump together and form a small, deeply stainable nucleus whereas they remain separate in the sperm-head (Fig. 31). A small proportion of the eggs are not entered by a spermatozoon, in these cases the second meiotic division takes place and two polar bodies are extruded. These eggs probably develop into the haploid embryos mentioned above. Fig. 32 shows the two true polar bodies from a haploid embryo, and in both nuclei the chromosomes clump together. It would, therefore, seem that the presence of spermatozoa suppresses the second meiotic division, and also that spermatozoa are required for the development of parthenogenetic eggs. In the other parthenogenetic species spermatozoa are also present in the cocoons, but it is unknown whether pseudo-fertilization is necessary for the successful development of the eggs in these cases.

C. Asexual reproduction

Asexual reproduction has only recently been recorded in the Enchytraeidae: in *Enchytraeus fragmentosus* by BELL (1959), in *Cognettia glandulosa*, *C. sphagnetorum* and *Buchholzia appendiculata* by CHRISTENSEN (1959). In *Enchytraeus fragmentosus* sexually mature worms have never been found whereas in the three latter species mature worms

occur for a short period during the cold seasons of the year. However, in *Cognettia sphagnetorum* asexual reproduction by fragmentation and regeneration is the only mode of reproduction since the eggs laid never hatch. *Cognettia glandulosa* and *Buchholzia appendiculata* are able to reproduce sexually as well as asexually. The regeneration of a new anterior end was briefly described by CHRISTENSEN (1959).

Section 3. Types of meiosis

In a relatively large number of Enchytraeid worms bivalent formation during the first meiotic division differs from normal. The following three main types of meiosis may be distinguished: 1) Chiasmata present (*chiasma-type*), 2) Chiasmata absent (*achiasma-type*), 3) No pairing between the homologues, but first division still reductional (*asynapsis-type*).

A. Chiasma-type

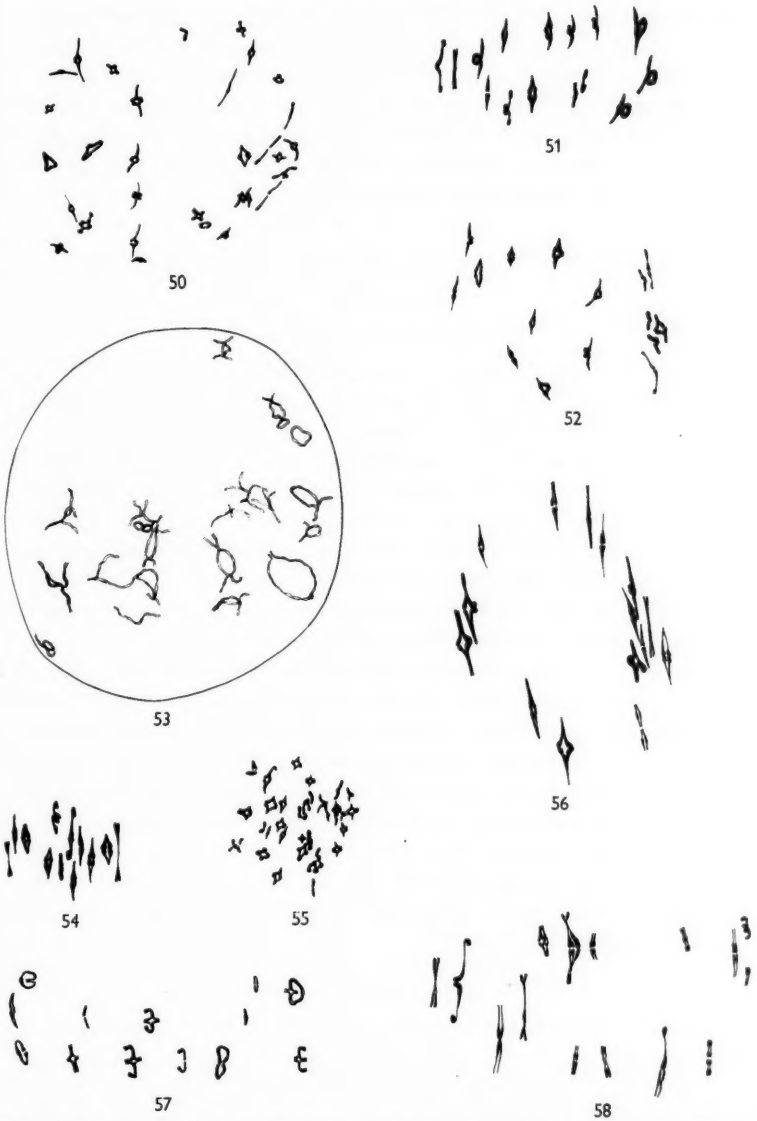
In the following genera and species the bivalents are of the *chiasma-type*. When nothing else is mentioned, the generic name indicates that all the species listed in Table 1 belong to this type:

Mesenchytraeus
Cernosvitoviella
Lumbricillus (except *L. lineatus 3x*)
Enchytraeus
Hemienchytraeus
Achaeta (except *A. bulbosa*)
Marionina (except *M. subterranea* and *M. sjaelandica*)
Bryodrilus
Henlea (except *H. perpusilla*)

Although, in all these species, homologous chromosomes are held together by chiasma(ta), there are some variations in such details as number and position of chiasmata and degree of contraction during the later stages of the meiotic prophase.

The smaller bivalents are usually fully terminalized when they become Feulgen positive and no differences occur between the species. Among the larger chromosomes, however, three main types of bivalents may be distinguished: *a*) both terminal and interstitial chiasmata, 2 or more chiasmata per bivalent, *b*) only (sub)terminal chiasma(ta), *c*) a single interstitial chiasma.

Figs. 38—49, oocyte MI, ×1500. — Fig. 38: *Henlea ventriculosa*; 39: *H. heleotropha*; 40: *H. similis*; 41: *H. nasuta*; 42: *Marionina argentea*; 43: *M. tubifera*; 44: *M. vesiculata*; 45: *Achaeta bohemica*; 46: *Marionina southerni*; 47: *M. spicula*; 48: *Lumbricillus viridis*; 49: *Cernosvitoviella atrata*.



Figs. 50—52 and 54—58, oocyte MI, ×1000; Fig. 53, oocyte prophase, ×800. — Fig. 50: *Achaeta affinis*; 51: *Marionina riparia*; 52: *Hemienchytraeus bifurcatus*; 53: *Enchytraeus capitatus*; 54: *Achaeta parva*; 55: *Achaeta danica*; 56: *Marionina communis*; 57: *Lumbricillus rivalis*; 58: *Bryodrilus ehlersi*.

In many species one of these types dominates among the larger bivalents, and in a few cases only one type is present. It is, therefore, in many cases possible to characterize genera, groups of related species and even single species by their bivalents. Representatives of the different types are described below; amphimictic and parthenogenetic species or cytotypes are considered separately, and a diploid and polyploid form is described when both exist.

a. *Bivalents with both interstitial and terminal chiasmata dominating*

Amphimictic forms

Diploids. — The oogenesis of *Lumbricillus lineatus* $2x$ as described in CHRISTENSEN (1960), may be taken as a representative of this type. The same type of bivalents are formed during spermatogenesis.

In the majority of diploid amphimictic Enchytraeidae the larger bivalents are probably of the present type. However, many species have not been available in sufficient number to enable a detailed analysis of the first meiotic prophase. The following species definitely belong to this category: *Mesenchytraeus flavus, M. beumeri, M. armatus, Cernosvitoviella atrata, Enchytraeus capitatus, Hemienchytraeus bifurcatus, Henlea cf. heleoptropha* and *Marionina riparia.*

Polyploids. — The following species may belong to this category: *Achaeta danica, A. bohemica, Henlea jutlandica* and *Marionina clavata.* It has, however, only been possible to make stainable preparations of the late meiotic prophase and the MI, and at these stages the bivalents are much contracted and leave no details as regards number and position of chiasmata. Some of the examples may, therefore, belong to one of the following categories with a reduced number of chiasmata. Oogenesis as well as spermatogenesis proceed in a regular way without formation of multivalents, and morphologically normal gametes of both types are produced.

Parthenogenetic forms

Diploids. — In the oocyte of *Mesenchytraeus glandulosus,* 16 typical bivalents are seen, Fig. 62. Chiasmata appear as a series of loops along most of the bivalents, while a few are already fully terminalized. In the late prophase the bivalents are much condensed and show no details as to number and position of the chiasmata. The anaphase disjunction, which in all members of the genus *Mesenchytraeus* takes place before

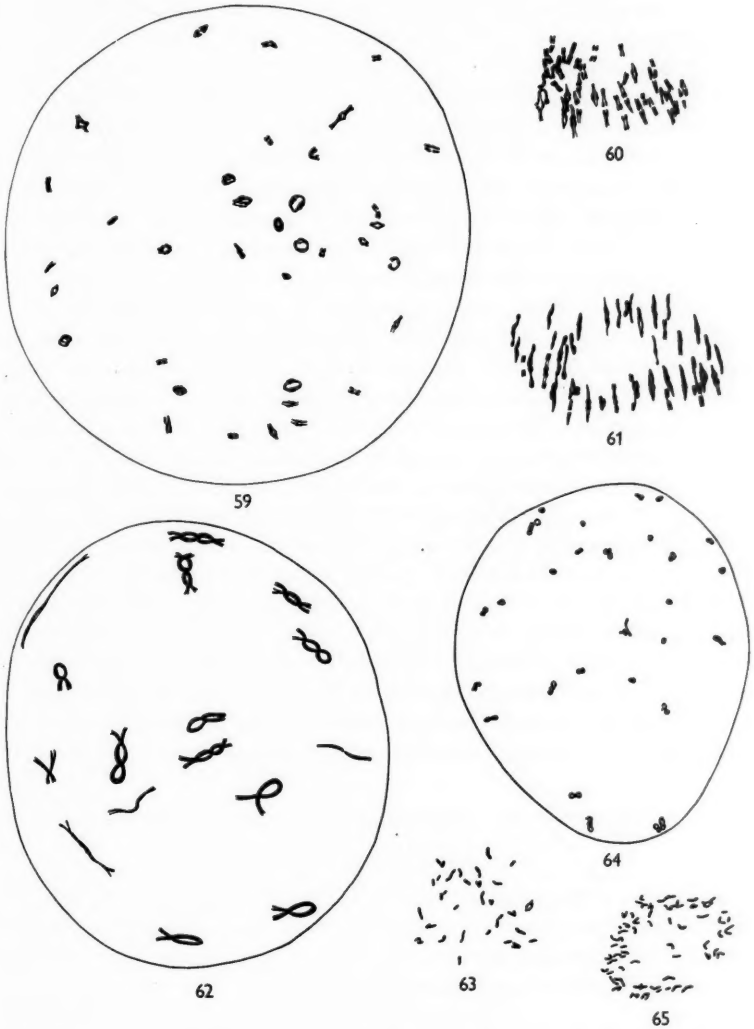


Fig. 59, *Henlea jutlandica*, oocyte prophase, apparently 36 bivalents, ×500. — Fig. 60, *H. jutlandica*, oocyte MI, ×1000. — Fig. 61, *Marionina clavata*, oocyte MI, ×830. — Fig. 62, *Mesenchytraeus glandulosus*, oocyte prophase, ×670. — Fig. 63, *M. glandulosus*, blastomere mitosis, ×670. — Fig. 64, *M. pelicensis*, oocyte prophase, ×400. — Fig. 65, *M. pelicensis*, blastomere mitosis, ×670.

the eggs are laid, is described on p. 426. At the first anaphase each of the two daughter nuclei receive a complete haploid set of chromosomes. During spermatogenesis the bivalents are too small to show any details as to number and position of chiasmata. At the first spermatocyte division 16 bivalents are seen, and the secondary spermatocytes receive a complete haploid set of chromosomes. Morphologically normal spermatozoa were present in all individuals examined. However, only about half the sexually mature worms contained spermatozoa in their spermathecae (worms from two different stations have been examined), hence a large proportion of the worms do not copulate.

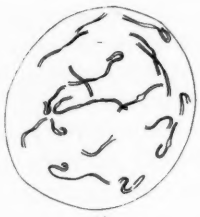
Polyploids. — In the oocyte prophase of *Mesenchytraeus pelicensis*, 32 typical bivalents are seen, Fig. 64. In the majority of the bivalents the homologues are held together by two or more chiasmata, multivalents have never been observed, and the distribution of chromosomes to the two daughter nuclei at the first meiotic anaphase is completely regular. Anaphasic disjunction of the homologues is described on p. 426. During spermatogenesis there is probably 32 bivalents at the first spermatocyte division. Morphologically normal spermatozoa are produced in the same number as in the diploid species of this genus. However, spermatozoa have never been found in the spermathecae of sexually mature individuals of *M. pelicensis* (worms from four different stations have been examined), it is, therefore concluded that the worms never copulate. In spite of this, spermatozoa are nearly always present in the cocoons, and they must be derived from the same worm which laid the eggs, whether some kind of self-activation is involved is unknown. *Achaeta affinis* probably also belong to this category.

b. *Bivalents with (sub)terminal chiasma(ta) dominating*

Amphimictic forms

Diploids. — The diploid species and cytotypes belonging to this category may be divided into two groups, in the first of these the majority of the larger metacentric bivalents have a single chiasma, in the second the larger bivalents have two chiasmata.

Figs. 66—71, *Enchytraeus coronatus*; Fig. 66: early oocyte prophase, ×500; 67: later oocyte prophase, ×500; 68: pro-metaphase, ×500; 69: oocyte MI, ×500; 70: spermatocyte MI, ×1000; 71: spermatogonial mitosis, ×1500. — Fig. 72—75, *Lumbricillus helgolandicus*; 72: early oocyte prophase, ×1000; 73: later oocyte prophase, ×1000; 74: spermatogonial mitosis, ×1500; 75: spermatocyte pro-metaphase, ×1000. — Fig. 76, *Lumbricillus tuba*, oocyte MI, ×1000. — Fig. 77, *L. kaloensis*, oocyte prophase, ×660.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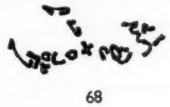
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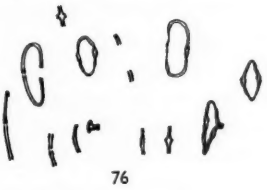
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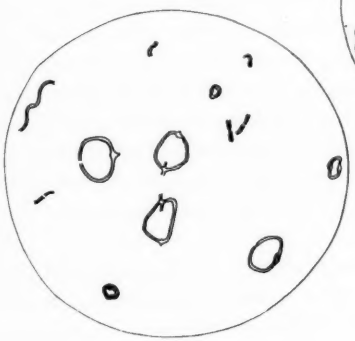
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In the primary oocyte of *Enchytraeus coronatus* n=18 the majority of the bivalents are held together by a single (sub)terminal chiasma from the earliest stainable stages of the prophase (Fig. 66). The homologues are arranged end to end, and the point of attachment is either a rather narrow part where chromatids are held close together, or, less frequently, the chromatids of each homologue separate laterally at or near the point of contact. This latter type may indicate the presence of a subterminal chiasma. The later stages of the prophase shows no change in the structure of bivalents other than a progressive shortening and thickening (Fig. 67), and the centromere region is not morphologically distinguishable during the prophase, but at pro-metaphase the larger metacentric homologues become V-shaped with the apices — the centromere regions — pointing in opposite directions (Fig. 68). At MI the chromosomes become much attenuated between centromere and chiasma (Fig. 69). Sometimes the nucleus proceeds into AI before the egg is laid.

The above description is based upon bivalents with a single terminal chiasma, by far the dominating type, but in addition bivalents with two terminal chiasmata and a single interstitial chiasma may occur. The spermatogenesis follows the same pattern as described for the oogenesis. Fig. 70 shows a spermatocyte pro-metaphase with the majority of bivalents held together by a single terminal chiasma, in addition there are two bivalents with two terminal chiasmata and one bivalent with a single interstitial chiasma.

Lumbricillus helgolandicus n=13 may be used as a representative of the species in which the larger chromosomes are held together by two terminal chiasmata. From the earliest Feulgen-positive stages of the oocyte-prophase, the bivalents are held together by terminal or almost terminal chiasmata, the larger usually by two, the smaller by one (Fig. 72). As prophase proceeds each homologue becomes visibly divided into two chromatids and a progressive shortening and thickening takes place (Fig. 73). In the three largest bivalents shown in this figure one of the two chiasmata is not strictly terminal. In the present species (and some of its relatives, see p. 425) there is hardly any diakinetic shortening of the chromosomes, the bivalents are, therefore, only slightly more contracted at MI than at diplotene.

Spermatogenesis follows the same pattern as described for the oogenesis. Fig. 75 shows a spermatocyte pro-metaphase from *L. helgolandicus* where six bivalents are ring-shaped with two terminal chiasmata and seven, mostly the smaller ones, are rod-shaped with a single termi-

nal chiasma. A few other species belong to this category: *Lumbricillus tuba, L. kaloensis* and some *Henlea*-species may have bivalents with primarily terminal chiasmata.

Polyploids. — A tetraploid cytotype of *Enchytraeus coronatus* has been found in a single station, from which it has later disappeared due to desiccation. The bivalents in the oocyte-MI had a single terminal chiasma and were much attenuated, exactly as in the MI of the diploid cytotype, and no multivalents were found. Morphologically normal spermatozoa were produced in large numbers.

Parthenogenetic forms

No parthenogenetic species are known in which bivalents with terminal chiasma(ta) dominate, but as will be mentioned below, such bivalents are present in the oocyte prophase of *Cognettia glandulosa.*

c. *Bivalents with a single interstitial chiasma dominating*

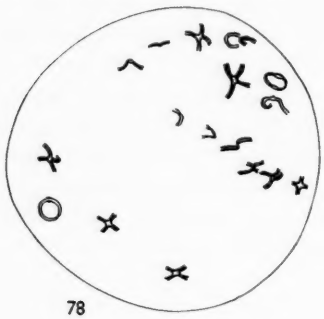
Amphimictic forms

Diploids. — The gametogenesis of *Enchytraeus buchholzi* n=18 is described as a representative of this type.

When the chromosomes become Feulgen-positive in the oocyte prophase, the majority of the larger bivalents are cross-shaped, indicating the presence of a single interstitial chiasma. As the prophase proceeds a gradual shortening and thickening of the bivalents takes place. At this time opposite arms are in most cases of equal length, and the centre of the cross is open, due to rotation of the chromatid pairs in relation to each other (Fig. 79). The extent to which the chiasma is terminalized seems to vary a great deal, in the MI the majority of the bivalents are in most cases still cross-shaped while in other cases the majority of the bivalents are held together by a terminalized chiasma already in late prophase. As the MI proceeds the chromosomes become attenuated and the chiasma gradually slides towards the ends. Usually one, less frequently two or three, of the larger bivalents have two terminal chiasmata. Spermatogenesis follows the same pattern as described for the oogenesis (Fig. 82).

Lumbricillus bülowi, Enchytraeus minutus and *E. lacteus* n=18 also belong to this category.

In a few other species the number of bivalents with a single interstitial chiasma is also high but their occurrence is not so constant as in



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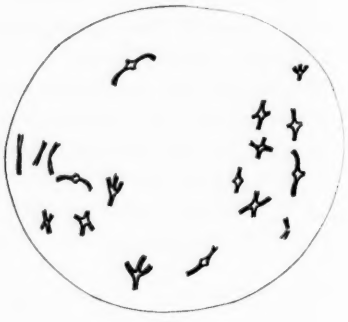

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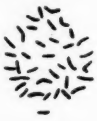

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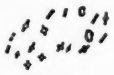

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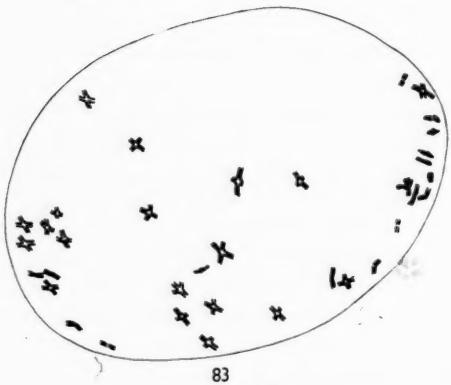

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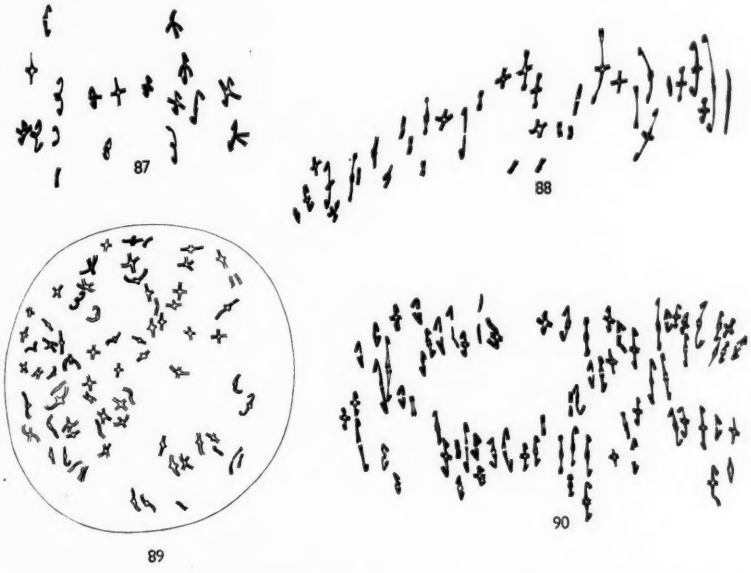
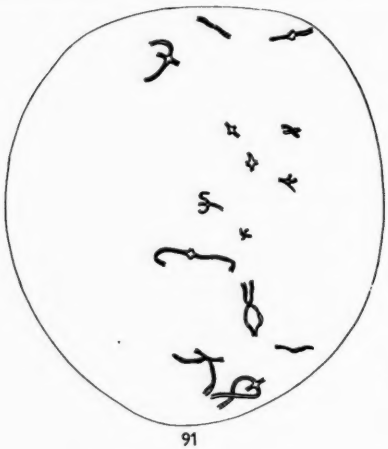
the species mentioned above: *Lumbricillus arenarius, L. pagenstecheri, Enchytraeus irregularis, E. albidus* and *Marionina appendiculata.*

Polyploids. — In the oocyte prophase of the tetraploid cytotype of *Enchytraeus buchholzi*, 36 bivalents are seen, the majority of the larger bivalents are cross-shaped with a single interstitial chiasma, the remaining are rod-shaped with a single terminal chiasma; bivalents with more than one chiasma have never been observed (Figs. 83 and 86). Spermatogenesis follows the same pattern as described for the oogenesis (Fig. 85). *Enchytraeus lacteus* n=36, c. 54 and 72 also belong to this category.



Figs. 87—90, *Enchytraeus lacteus*; Fig. 87: oocyte MI n=18, ×1000; 88: oocyte MI n=36, ×1000; 89: oocyte prophase n=72, ×800; 90: oocyte MI n=72, ×1000.

Figs. 78—82, *Enchytraeus buchholzi* n=18; Figs. 78—79; oocyte prophases, ×1000; 80: oocyte MI, ×1000; 81: spermatogonial mitosis, ×1500; 82: spermatocyte MI, ×1000. — Figs. 83—86, *E. buchholzi* n=36; Fig. 83: oocyte prophase, ×1000; 84: spermatogonial mitosis, ×1500; 85: spermatocyte MI, ×1500; 86: oocyte MI, ×1200.



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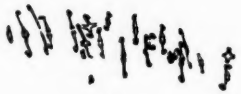

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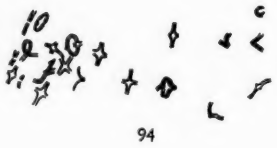

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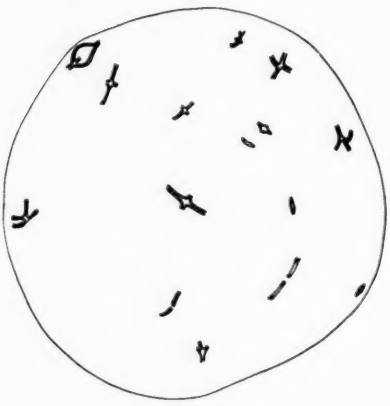

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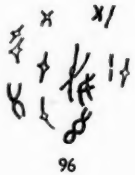

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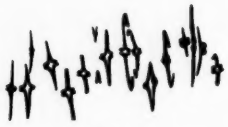

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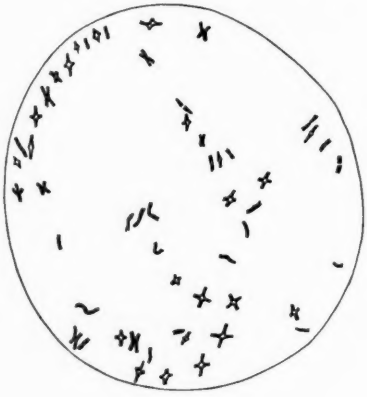

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Parthenogenetic forms

Diploid parthenogenetic species with this type of bivalents are not known. In the oocyte prophase of the parthenogenetic polyploid species *Cognettia glandulosa* the haploid number of bivalents is seen. Most of the homologues are held together by a single interstitial chiasma, the remaining bivalents are arranged end to end with a single terminal chiasma, bivalents with more than one chiasma, and multivalents have never been observed (Fig. 99). The distribution of the chromosomes to the two daughter nuclei at the first meiotic anaphase is fully regular.

In a few individuals spermatozoa are completely absent, but the majority of the worms produce morphologically normal spermatozoa in large numbers. At the first spermatocyte division the "haploid" number of bivalents is apparently formed (due to the high numbers involved accurate counts have not been possible), and the distribution of the chromosomes to the two daughter nuclei at the first anaphase is apparently regular.

In both cytotypes of *Cognettia sphagnetorum* bivalents of the same types as in *C. glandulosa* are seen in the oocyte prophase, but, the eggs laid do not complete development (cf. p. 403 and CHRISTENSEN, 1959).

B. Achiasma-type

In the following genera and species the bivalents are of the *achiasma*-type. When only the generic name is given, it indicates, that all species listed in Table 1 belong to this type:

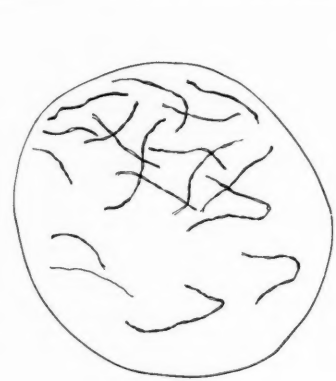
Fridericia
Buchholzia
Hemifridericia
Enchytronia parva
Achaeta bulbosa
Marionina subterranea and *M. sjaelandica*
Henlea perpusilla

Fig. 91, *Lumbricillus pagenstecheri*, oocyte prophase, ×1000. — Fig. 92, *L. arenarius*, oocyte MI, ×1000. — Fig. 93, *Enchytraeus albidus*, oocyte MI, ×1000. — Fig. 94, *E. irregularis*, oocyte pro-metaphase, ×1000. — Fig. 95, *Lumbricillus bülowi*, oocyte prophase, ×1000. — Fig. 96, *L. bülowi*, spermatocyte pro-metaphase, ×1000. — Fig. 97, *Marionina appendiculata*, oocyte MI, ×1000. — Fig. 98, *Enchytraeus minutus*, oocyte MI, ×1000. — Fig. 99, *Cognettia glandulosa*, oocyte prophase, ×660.

During the meiotic prophase, the homologous chromosomes are held together without formation of chiasmata. The homologues are parallelly paired throughout their length during the entire prophase, and there is no trace of the opening-out process of the orthodox diplotene-diakinetic stage (cf. Figs. 100—102 and 121—122). When the spindle forms, the bivalents, still unopened, move to the equator, while maintaining an orientation parallel to the long axis of the spindle. When the bivalents are arranged in the metaphase plane, the centromeres move coincidently toward opposite poles. Anaphase disjunction begins with a uniform separation of the homologues throughout their length. During this disjunction sister chromatids are closely associated, and no interchromosomal crossing of strands are disclosed (cf. Figs. 103 and 123). When the homologues are brought into complete terminal opposition, or have separated, the two chromatids of each chromosome become optically separable (cf. Figs. 108 and 115). In the mitotic metaphase plate there is no special attraction between homologous chromosomes (somatic pairing), cf. Figs. 104 and 107, but in the early mitotic anaphase of *Fridericia ratzeli* strong attraction between the daughter chromosomes causes the separation to extend gradually from the centromere region towards the end(s) of the chromosome, and a metacentric chromosome becomes increasingly V-shaped as its centromere moves toward the pole (Fig. 111). The late meta- and early anaphase stages of meiotic and mitotic divisions are, therefore, morphologically very similar although, of course, in the former case the chromosome complements consist of two homologous chromosomes while in the latter case two daughter chromosomes are present.

On a morphological basis the *achiasmatic* bivalents may be divided into three groups, for convenience named: 1) the *Fridericia-type*, see also VEJDOVSKY (1907), 2) the *Marionina subterranea-type* and 3) the *Henlea perpusilla-type*. This latter type is less clearly defined than the two former. Representatives of the different types are described in greater detail below, amphimictic and parthenogenetic species or cytotypes are considered separately, and a diploid and polyploid form is described when both exist.

Figs. 100—105, *Buchholzia fallax* n=18; Fig. 100: early oocyte prophase, ×1000; 101: later oocyte prophase, ×1000; 102: late oocyte prophase, ×1000; 103: oocyte MI, ×1250; 104: spermatogonial mitosis, ×1500; 105: spermatocyte MI, ×1250. — Figs. 106—107, *B. fallax* n=36; Fig. 106: oocyte MI, ×1250; 107: spermatogonial mitosis, ×1500.



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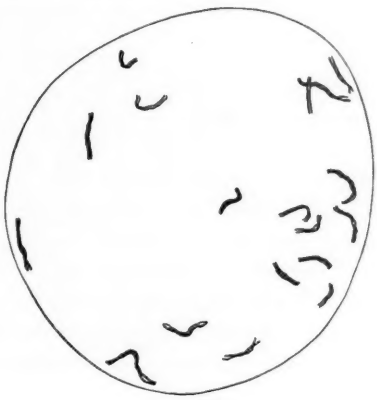

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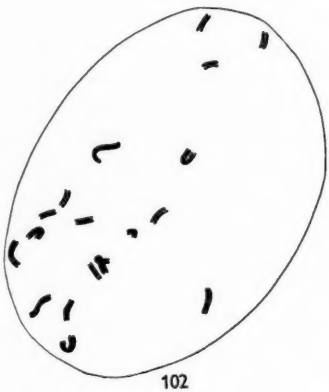

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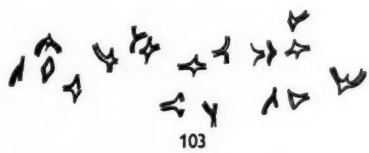

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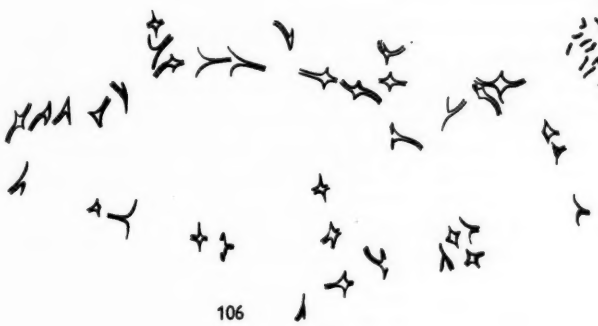

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a. *The Fridericia-type*

Amphimictic forms

Diploids. — In the early oocyte prophase of the diploid cytotype of *Buchholzia fallax* homologous chromosomes show parallel pairing throughout their entire length and they are so closely associated as to appear as a single thread (Fig. 100). As prophase proceeds a progressive shortening and thickening of the bivalents takes place, and at the same time the homologues become less closely paired so that now the two homologues of each bivalent are morphologically distinct (Fig. 101). A bivalent appears in the late prophase as two parallel rods without constrictions to indicate the position of the centromere regions, and there is no repulsion at any point along the length of the homologues (Fig. 102).

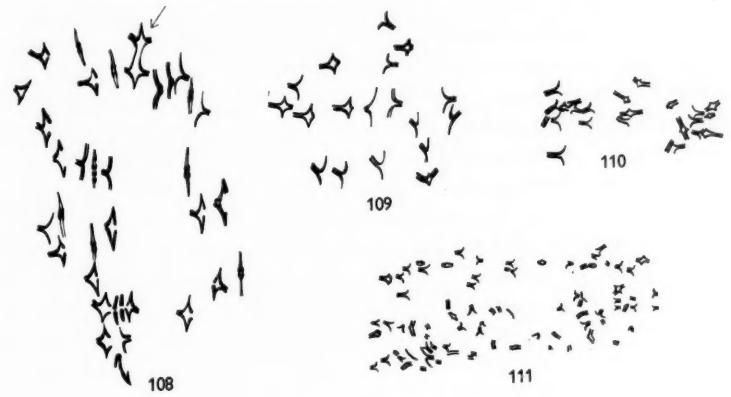
When the bivalents are arranged in the MI plane the centromeres move towards the poles and the two homologues of each bivalent are gradually drawn apart (Fig. 103). The diameter of a chromosome gradually increases from the centromere towards the paired region of each arm, and the paired regions retain the precisely parallel and equidistant position characteristic of the prophase. As the MI proceeds the centromeres move further apart and the smaller acrocentric chromosomes are brought into precise terminal apposition while the larger metacentric chromosomes are gradually drawn out into opposing V's.

The bivalent formation during the spermatogenesis follows the same pattern as described for the oogenesis. The homologues show parallel pairing throughout the spermatocyte prophase, and at MI the bivalents appear as two closely associated parallel rods with a slight opening out at and near the centromere region.

Other species belonging to this category: *Buchholzia appendiculata* and *Enchytronia parva.*

Polyploids. — In the oocyte prophase and metaphase of the tetraploid cytotype of *Buchholzia fallax*, the bivalents behave exactly as described for the diploid cytotype above. Among a total of 22 cells analyzed, only 5 quadrivalents were observed, two from prophases, three from MI, and

Fig. 108, *Buchholzia fallax*, tetraploid cytotype, oocyte MI, tetravalent indicated by an arrow, ×1250. — Fig. 109, *Buchholzia appendiculata*, oocyte MI, ×1250. — Fig. 110, *Enchytronia parva*, oocyte MI, ×1000. — Fig. 111, *Fridericia ratzeli* n=40, late metaphase from blastomere mitosis, ×1000. — Fig. 112, *Buchholzia fallax*, apparently octoploid oocyte pro-metaphase from an otherwise tetraploid individual, ×1250. — Fig. 113, *Henlea perpusilla* n=17, oocyte prophase, ×660. — Fig. 114, *H. perpusilla* n=34, oocyte prophase, ×860.



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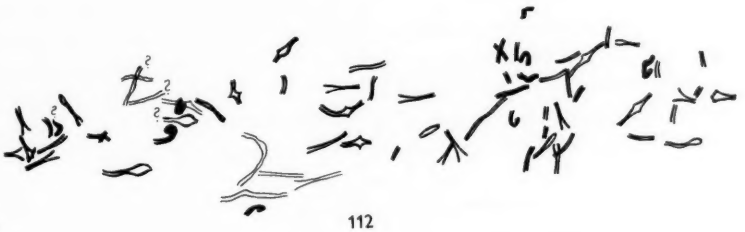

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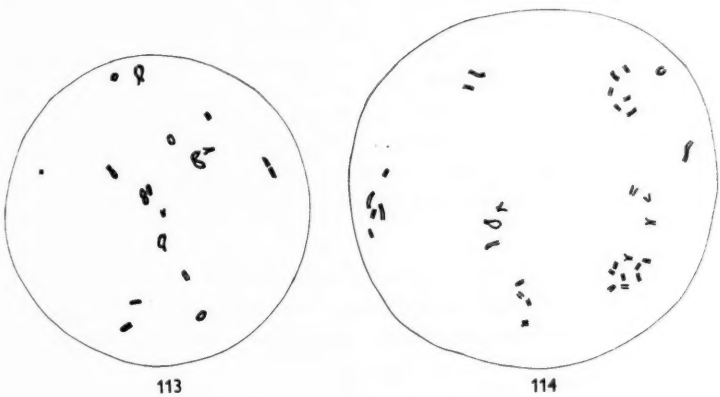

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they all consisted of meta- or submetacentric chromosomes. In the prophase the four homologues are seen to be associated in a cross with but slight central aperture. Each of the four arms is strictly comparable in structure to one arm of a normal bivalent of the same type. Superficially these prophase quadrivalents resemble the open cross tetrad formed by the rotation of chromatid pairs in an ordinary bivalent with one chiasma, each chromosome simulating a chromatid. In the three quadrivalents observed in MI, only two centromeres (opposite) were drawn towards the poles, and the four homologues appeared like two crosses held together by the two remaining centromeres (Fig. 108). This metaphase orientation probably results in a regular anaphase distribution when the two arms of the chromosomes in question are of different length, since the shorter arms will separate before the longer ones.

In a ordinary tetraploid individual a single unusually large egg was found. A cytological investigation revealed an early MI (Fig. 112), in which some bivalents still maintained an orientation parallel to the long axis of the spindle, while others were arranged in the metaphase plane, but only few showed an opening out in the centromere region. The preparation was not completely successful, since some chromosomes were clumped together, but 64 bivalents were distinguishable, and the nucleus in question was undoubtedly octoploid. No multivalents were observed.

Due to the large number and small size of chromosomes a detailed analysis of the spermatogenesis has not been possible, but the first meiotic division appears to be regular with the formation of the "haploid" number of bivalents. Morphologically normal spermatozoa are produced to the same extent as in the diploid cytotype of *B. fallax*.

Other species and cytotypes belonging to this category: *Fridericia bulbosa*, *F. bulboides*, *F. callosa*, *F. striata*, *F. paroniana*, *F. bisetosa* n=32, *F. perrieri*, *F. galba*, *F. gracilis*, *F. maculata* n=26, *F. leydigi* n=32, *F. connata* n=32 and 64, *F. hegemon*, *Hemifridericia parva* n=28 and c. 42.

Parthenogenetic forms

No diploid parthenogenetic species with this type of bivalents are known. The gametogenesis of the polyploid *Fridericia ratzeli* is described as a representative of this type.

Fig. 115, *Fridericia bisetosa* n=56, oocyte MI, ×1000. — Fig. 116, *F. connata* n=56, oocyte MI, ×1000. — Fig. 117, *F. maculata* n=45, oocyte MI, ×1000. — Fig. 118, *F. ratzeli* n=56, oocyte prophase, ×415. — Fig. 119, *F. ratzeli* n=40, oocyte prophase, ×415. — Fig. 120, *Achaeta bulbosa*, oocyte MI, ×900.

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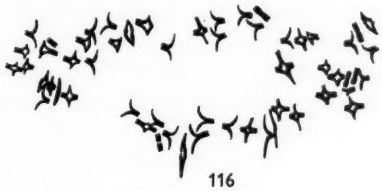

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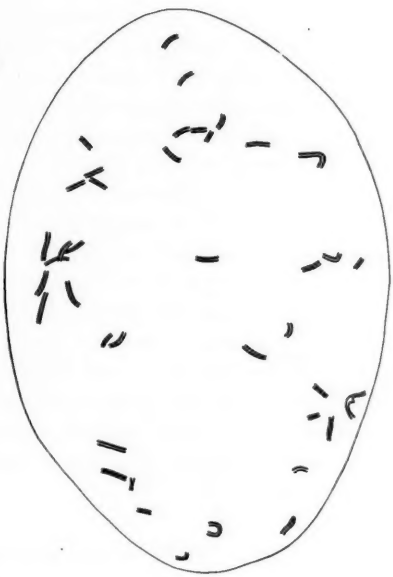

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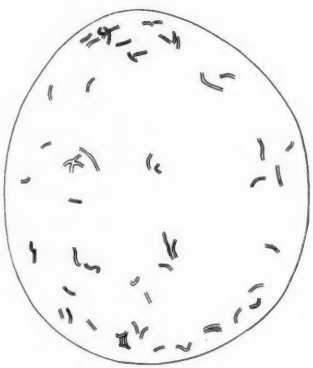

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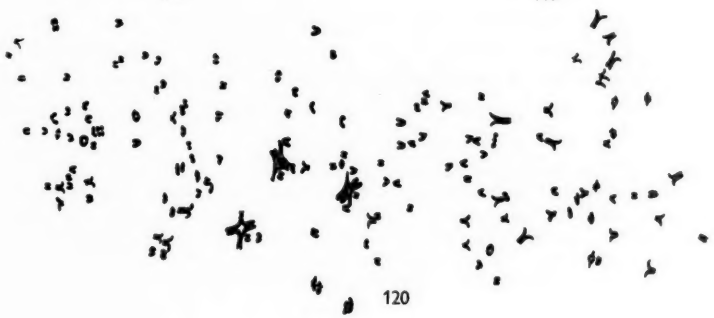

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Throughout the oocyte prophase the homologues show completely parallel pairing as described above for the amphimictic species (Figs. 118 and 119) and at the MI—AI the same gradual separation of the homologues takes place. In the oocyte prophase of the n=56 cytotype some quadrivalents are occasionally formed with a frequency that varies between cells, but more than three in a single cell have never been observed. The orientation of the four centromeres at MI has not been observed, it is, therefore, unknown whether the distribution of the chromosomes to the two daughter nuclei at AI is regular in all cases.

Some individuals does not produce mature spermatozoa. The development of the male cells is arrested at a rather early stage in these cases, and a first spermatocyte division with the "haploid" number of bivalents has never been observed. In spite of this spermatid-like cells may occur, but never mature spermatozoa. In other individuals spermatogenesis proceeds normally, and mature spermatozoa are produced. Worms belonging to either type occur in the same habitat, and although the eggs develop parthenogenetically spermatozoa are always present in the spermathecae of sexually mature individuals (also those which produce no spermatozoa themselves). The possible rôle of the spermatozoa in the activation of the eggs remains to be studied.

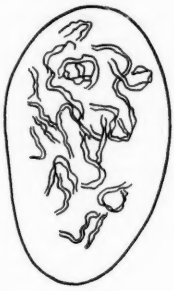
The following species and cytotypes probably also belong to this category: *Fridericia connata* n=56, *F. bisetosa* n=56, *F. maculata* n=45, *F. leydigi* n=60(?) and *Achaeta bulbosa*(?). *Fridericia bisetosa* n=56 differs from the other cytotypes through that the majority of homologues are at once brought into terminal apposition in the first meiotic metaphase, and often connected by a thin thread of chromatin material (Fig. 116).

b. *The Marionina subterranea-type*

Only diploid amphimictic species are known with this type of bivalents, namely: *Marionina subterranea* and *M. sjaelandica.*

From the earliest stages of the oocyte prophase the homologues chromosomes show parallel pairing (Fig. 121), but in contrast to the *Fridericia*-type the homologues of each bivalent are always visibly

Figs. 121—125, *Marionina subterranea*; Fig. 121: early oocyte prophase, ×1250; 122: late oocyte prophase, ×1250; 123: oocyte MI, ×1250; 124: spermatocyte MI, ×1500. — Fig. 125, *Marionina sjaelandica*, oocyte MI, ×1500. — Figs. 126—127, *Henlea perpusilla* n=17, ×1500; Fig. 126: oocyte MI; 127: oocyte AI. — Figs. 128—129, *Mesenchytraeus armatus*, ×1250; Fig. 128: oocyte prometaphase; 129: oocyte AI. — Figs. 130—131, *M. pelicensis*, ×1250; Fig. 130: oocyte MI—AI; 131: oocyte AI.



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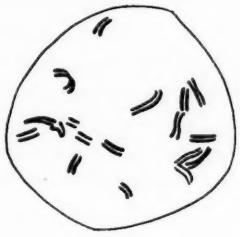

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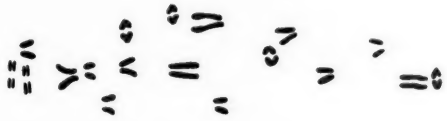

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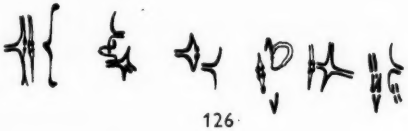

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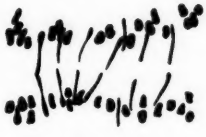

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separated in the present type. The occasional connections between the two homologues seen at this stage are probably not chiasmata, but rather artefacts produced during fixation. As the prophase proceeds a shortening and thickening of the chromosomes takes place, and in the late prophase the bivalents appear as two parallel rods, seemingly without connections, and without repulsion or a morphologically differentiated centromere region (Fig. 122). The distance between the two homologues of a bivalent is relatively larger in the present type than in the *Fridericia*-type, approximately equal to the diameter of the homologous chromosomes.

When arranged in the first meiotic metaphase plane the two centromeres of each bivalent begin to separate. This is most conspicuous in the smaller and medium sized bivalents, in which the two homologues are gradually drawn apart (Fig. 123). In the largest metacentric bivalents, however, there is only a slight separation in the centromere region, whereas the distal parts of the arms remain parallel throughout the whole metaphase stage.

Bivalent formation during spermatogenesis follows the same pattern as described for the oogenesis. The homologous chromosomes are parallelly paired and visibly separated throughout the spermatocyte prophase, in the MI they appear as two parallel rods (Fig. 124).

c. *The Henlea perpusilla-type*

Henlea perpusilla is the only species with this type of bivalents, it comprises a diploid cytotype in which the nucleus is in the first meiotic anaphase (AI) when the eggs are laid, and a tetraploid cytotype which may be heterogenous since in some populations the nucleus is in AI in others in MI when the eggs are laid. The anaphasic disjunction of the homologues where the nucleus is in AI when the eggs are laid, is described on p. 426. Both cytotypes are apparently amphimictic.

Diploids. — In the earlier stages of the oocyte prophase of the diploid cytotype, the homologous chromosomes are parallelly paired and visibly separated in the majority of the bivalents, in others the homologues show connections at some points indicating the presence of chiasmata (Fig. 113). However, the oocyte prophase of the present species — and most other members of the genus *Henlea* — are not suitable for cytological investigations because the bivalents are difficult to fix properly and they stain rather weakly, the connections between the homologues may, therefore, be artefacts. In a few bivalents the homologues are

apparently arranged end to end, and these are probably held together by a single, completely terminalized, chiasma. Occasionally there are some univalent chromosomes in the late oocyte prophase. Since bivalents have only been found at the oocyte MI and since the chromosomes are regularly distributed to the two daughter nuclei at the first meiotic anaphase some kind of "toach-and-go" mechanism must be involved, but the problem needs further investigation. The bivalents of the spermatocyte divisions are too small to allow any detailed analysis. The haploid number of bivalents is formed and morphologically normal spermatozoa are produced.

Polyploids. — The oocyte prophase of the tetraploid cytotype of *H. perpusilla* shows the same variable picture as described in the diploid, bivalents with and without chiasmata occur (Fig. 114). No multivalents have been observed, and the distribution of the chromosomes to the two daughter nuclei at the first meiotic anaphase is completely regular. A detailed analysis of the spermatogenesis has not been possible, but the first meiotic division appears to be regular with formation of the "haploid" number of bivalents. Morphologically normal spermatozoa are produced to the same extent as in the diploid cytotype.

C. Asynapsis-type

In the triploid (3x=39) parthenogenetic cytotype of *Lumbricillus lineatus*, the first meiotic division is asynaptic and no typical metaphase plate is formed, the univalents are distributed to opposite poles without undergoing an equational division. The first division is, therefore, numerically reductional, for further details, see CHRISTENSEN (1960).

Section 4. Diakinetic contraction and anaphasic disjunction

In this section some differences between the genera in the degree of contraction of the bivalents in the late prophase are pointed out, furthermore chromosome behaviour is described in species where the nucleus proceeds to the first meiotic anaphase stage before the egg is laid.

Concerning the diakinetic contraction of the bivalents in the oocyte prophase two extreme types are clearly distinguishable. In one of these the chromosomes are highly contracted and appear as small deeply stained knobs which leave no details as to number and position of chiasmata in late prophase. This type is characteristic of *Mesenchytraeus* species (cf. Fig. 128, which shows a pro-metaphase of *M. arma-*

tus). In the other type the diakinetic shortening of the chromosomes is not very pronounced, the homologues are visibly divided into two chromatids, and the number and position of chiasmata are clearly seen (Figs. 73, 77, 79 and 91). This type occurs in most members of the genera *Lumbricillus* and *Enchytraeus*. Other Enchytraeidae occupy an intermediate position between these two extremes.

In the majority of Enchytraeidae the MI stage of the oocyte persists until the eggs leave the worm. To some extent the duration of the MI stage depends on environmental conditions, at low temperatures, when the production of cocoons is at a minimum, it may last for several months. In a few species, however, the MI stage is of a short duration and proceeds into AI. The eggs are deposited with the chromosomes in this anaphase stage, which may also last for several months. There is some differences in the behaviour of the chromosomes in the oocyte prophase of those species which deposit eggs in AI, three types are distinguishable:

(1) In *Lumbricillus lineatus 3x* the first meiotic division is asynaptic without formation of bivalents and no typical metaphase plate is formed, the univalents are distributed to opposite poles without undergoing an equational division (CHRISTENSEN, 1960).

(2) In all *Mesenchytraeus* species investigated, the chromosomes are in AI when the eggs are deposited. In the oocyte prophase the haploid number of typical bivalents with chiasmata are seen. In late prophase the chromosomes are highly contracted and when the nuclear membrane breaks down and the bivalents move towards the equatorial plane they appear as small deeply staining knobs. As soon as they are arranged in the metaphase plate the two centromeres of each bivalent are pulled apart towards opposite poles, the two homologues become much attenuated (Fig. 130) and are brought into complete terminal opposition. This metaphase stage is of very short duration; the homologues separate almost immediately, afterwards they contract to the same extent as in the late prophase prior to the metaphasic stretch (Figs. 129 and 131). The metaphase stage, with attenuated chromosomes, seems to last longer in the tetraploid *M. pelicensis* than in the diploid species.

(3) *Henlea perpusilla* comprises two (three) cytotypes (cf. Table 1), in n=17 AI (and n=34 AI), the eggs are in the first meiotic anaphase (AI) when deposited. The oocyte prophase of both cytotypes show a rather variable picture as described on p. 424. Bivalents with and without chiasmata occur, and occasionally some univalents are present.

When the chromosomes are arranged in the metaphase plate the homologues are gradually drawn apart towards opposite poles, during this process they become slightly attenuated, and eventually they separate, but in contrast to the former type, the chromosomes do not contract immediately upon the separation. The MI stage lasts considerably longer in this type than in the *Mesenchytraeus* species (Figs. 126 and 127).

In *Lumbricillus* and *Henlea* the behaviour of the chromosomes in the oocyte prophase — absence of bivalents and incomplete bivalent formation — may "explain" why the MI proceeds directly into anaphase. In the *Mesenchytraeus* species, however, the first meiotic division is completely normal, and typical bivalents are formed in which the homologues are held together by several chiasmata.

The combination of normal bivalent formation, short duration of MI and eggs in AI when deposited is characteristic of five *Mesenchytraeus* species investigated, hence it may turn out to be a useful generic character. The short duration of the MI stage in this genus is probably a specialized character.

IV. DISCUSSION

1. The evolution of Enchytraeidae based upon morphological characters

Although it is difficult to assign any definite position to the Enchytraeidae in relation to other Oligochaete families, it is generally agreed that they are related to the aquatic families Tubificidae, Naididae and Phreodrilidae. These are the ancestral, the Enchytraeidae the derived group. Similarity to these families must, therefore, be considered primitive characters in Enchytraeidae.

The presence in the genera *Mesenchytraeus* and *Cernosvitoviella* of setae with nodulus, nephridia without interstitial tissue, atrium-like expansion of the sperm duct, and free spermathecae (in some species) are no doubt inheritances from tubificid-like ancestors, and the two genera are undoubtedly the most primitive dealt with here. In addition *Mesenchytraeus* has a sperm- and egg-sac of the same type as in the Tubificidae.

The relationship between the remaining genera are somewhat doubtful, mainly because the detailed structure and function of various appendages connected with the alimentary canal, is only little known. It can, however, be stated that presence of these structures is the sequen-

tial and absence the antecedent condition. The genera *Cognettia* and *Lumbricillus*, where such appendages are absent, are, therefore, rather primitive. In this connection it is interesting to note, that the genera and species already mentioned are adapted to littoral and other permanently wet habitats. It is probably in this zone that the Enchytraeidae did separate from their aquatic ancestors, and these habitats are, therefore, the ancestral, from which the worms have invaded the terrestrial habitats which now harbour the majority of species. The genera *Fridericia*, *Hemifridericia* and *Buchholzia* have two kinds of lymfocytes, large granulated and small hyaline ones. If the latter type is of the same nature in the three genera it probably reflects kinship. In *Hemienchytraeus* and *Enchytraeus* the peptonephridia (salivary glands) are of quite similar structure, and the two genera are certainly related. *Bryodrilus* and *Henlea* have the same type of spermatheca and lymfocytes and the nephridia are of the same shape. The position of the genera *Achaeta* and *Enchytronia* is unknown while *Marionina* is very heterogenous, comprising several groups of species only remotely related between them. According to CERNOSVITOV (1937) *Achaeta* is related to *Hemienchytraeus*. How the various groups of genera are related *inter se* is not known at present.

2. The evolution of chromosome numbers

It will appear from the histogram in Fig. 132, which shows haploid chromosome numbers in Enchytraeidae (black columns), that a peak occurs between 12 and 21, with less pronounced maxima at even numbers between 32 and 36 and perhaps at 54 and 56. The two latter peaks comprise exclusively polyploids (cf. pp. 396 ff.) the first tetraploids, in the second *Enchytraeus lacteus* n=c. 54 is hexaploid whereas the exact degree of the remaining cytotypes is unknown.

The haploid chromosome numbers and number of cytotypes are summarized in the text-table below, to which reference is made throughout the following discussion on the basic numbers *within* the different genera:

Haploid chromosome numbers in the genera of Enchytraeidae and number of cytotypes (in brackets).

Genus	Haploid numbers (number of cytotypes)
Mesenchytraeus	16(4), 32(1)
Cernosvitoviella	13(1)
Cognettia	c. 54(2), c. 160(1)

Genus	Haploid numbers (number of cytotypes)
Lumbricillus	13(5), 14(2), 15(2), 17(1)
Enchytraeus	16(1), 18(3), 21(3), 36(3), c. 54(1), 72(1)
Hemienchytraeus	16(1)
Achaeta	12(1), c. 18(1), c. 27(1), 32(1), 36(1), 150—160(1)
Enchytronia	24(1)
Fridericia	26(1), 32(12), 40(1), 45(1), 56(3), 60(1), 64(1), 80—90(1)
Buchholzia	18—19?(2), 36—38?(1)
Hemifridericia	28(1), c. 42(1)
Henlea	16(1), 17(4), c. 34(2)
Bryodrilus	15(1)
Marionina	15(2), 17(3), 18(4), 20(1), c. 24(1), c. 38(1)

Mesenchytraeus: Basic number n=16.

Cernosvitoviella: Basic number n=13 ?.

Cognettia: The chromosome numbers known are all polyploid, and the basic number of the genus is not known (cf. p. 396).

Lumbricillus: The most primitive species have n=15 and 17, the sequential ones n=13 and 14. The basic haploid number of the genus is therefore probably 15, 16? or 17, consequently a decrease in chromosome number has taken place during the evolution of the genus.

Enchytraeus: Based upon morphological criteria it is difficult to assign which species are antecedent and which are sequential. But *Hemienchytraeus* and *Enchytraeus* are related (cf. p. 428), and the occurrence of n=16 in *Hemienchytraeus bifurcatus* and *Enchytraeus minutus* suggests that this is the basic number of the present genus. Consequently an increase in chromosome number has taken place with speciation at the n=18 level and at n=21.

Hemienchytraeus: Basic number n=16?.

Achaeta: The morphologically most primitive species *A. camerani* has n=c. 18, and the basic number of the genus may be at this level. Consequently both decrease (*A. parva* n=12) and increase in chromosome numbers have taken place.

Enchytronia: Basic number n=24?.

Fridericia: The majority of species have n=32, and it is likely that speciation within the genus has taken place at this level. There are, however, reasons to suspect the species in question to be tetraploids (cf. p. 396) probably derived from an ancestral basic number of 16.

Buchholzia: Basic number n=18 (19?).

Hemifridericia: The haploid numbers 28 and c. 42 are known. These are both multiples of 14, which may be the basic number of the genus.

Henlea: H. cf. heleotropha n=16 do not possess intestinal diverti-

culae, and is probably primitive within the genus; n=16 may, therefore be the ancestral number. Consequently an increase in chromosome number to n=17 has taken place during the evolution of the genus.

Bryodrilus: Basic number n=15?.

Marionina: Due to heterogeneity the concept of basic numbers cannot be applied to this genus.

Apart from polyploids the evolution of the family has taken place largely at the haploid level between 12 and 21. As mentioned in the preceding section, the genus *Mesenchytraeus* is reasonably suspected to occupy a particularly primitive position in relation to the remaining genera from which cytological information is available. The basic haploid chromosome number in this genus is 16, which is also likely to be the basic number in three other genera (*Enchytraeus*, *Hemienchytraeus*, *Henlea*), and the *Fridericia* species with n=32 are considered as tetraploids, derived from an ancestral basic number of 16. This means, that 1) n=16 is the only number recorded in the most primitive genus, 2) it is probably the basic number in 5 genera, and 3) n=16 and multiples thereof are by far the most common haploid numbers within the family. It seems, therefore, reasonable to assume n=16 to be the ancestral haploid number within the family, and that all other numbers, excepting polyploids, have been derived by non-polyploid evolution.

On the assumption that n=16 is the ancestral haploid number of the family, increase and decrease in number must have taken place several times during the evolution of the family. All haploid numbers up to 24 are probably of non-polyploid origin, whereas n=26 and higher numbers are polyploids (cf. p. 398); 47 cytotypes are then basicly diploid, and among these 8 cytotypes have n=16, 15 below and 24 above this number. In most cases, therefore, the number of chromosomes has been increased, and among the 15 cytotypes with less than 16 chromosomes in a haploid set, no less than 9 belong to the genus Lumbricillus, and only in this genus has there been a general trend towards reduction of the number of chromosomes, from morphologically more primitive species with n=17 and 15 (*L. arenarius* and *bülowi*) to more advanced forms with n=13 and 14 (i.e. *L. lineatus* and *pagenstecheri*).

When compairing basic numbers with the relationship between the genera as outlined on p. 427, it is seen: 1) that groups of related genera may have the same basic number, i.e. *Enchytraeus* and *Hemienchytraeus*, or different basic numbers, i.e. *Fridericia*: 32 (16?), *Buchholzia*: 18 (19) and *Hemifridericia*: 14, 2) there may be a slight tendency for

the more primitive genera (*Mesenchytraeus, Cernosvitoviella* and *Lumbricillus*) to have relatively low chromosome numbers, n=16 or below.

To sum up, therefore, we may say that the wide range of haploid numbers found in this family, reflects both intra- and intergeneric variation, and at the inter-generic level no clear unidirectional tendency towards either high or low chromosome numbers is demonstrable. At the intra-generic level, however, it is in some cases possible to demonstrate directional tendencies, e.g. against lower chromosome numbers in the genus *Lumbricillus* and against higher numbers in *Enchytraeus*.

Among diploid amphimictic earthworms (Lumbricidae) only 5 different haploid numbers are known: 11, 15, 16, 17 and 18 (MULDAL, 1952; OMODEO, 1952, 1955), whereas among diploid Enchytraeidae at least 10 different numbers occur: 12—18 (19?), 20—21 and 24. Although the much higher number of species should not be forgotten, it can be stated that the numbers of chromosomes vary much more in Enchytraeidae than in Lumbricidae.

OMODEO (1952) considers n=18 to be the ancestral number of the Lumbricidae from which the others have been derived, probably by centromere fusion in some chromosomes. According to OMODEO, therefore, there has been a general trend towards lower chromosome numbers in the earthworms, whereas in Enchytraeidae both increase and decrease have taken place.

3. Evolution of meiotic mechanisms

Three main types of meiosis are observed: 1) *Chiasma-type*, 2) *Achiasma-type* and 3) *Asynapsis-type*, and within the two former some variations occur which are described on p. 403 et sq.

The *chiasma-type* is the usual type of meiosis, and the ancestral type in the present family, which in some cases has been replaced by the two other kinds of mechanisms. Within the *chiasma-type* some variations occur being as to such details as the number of chiasmata per bivalent and their localization in particular regions. In the primitive genera *Mesenchytraeus* and *Cernosvitoviella* most of the bivalents have several chiasmata each, and this is, as one would expect, the ancestral type within the family, and any reduction in the number of chiasmata per bivalent is sequential. Two types of bivalents with a reduced number of chiasmata may be distinguished (cf. pp. 408 and 411). In the following species bivalents with primarily (sub)terminal chiasma(ta) dominate: *Enchytraeus coronatus, Lumbricillus helgolandicus, L. tuba, L. kaloensis* and perhaps some *Henlea* species. Bivalents with a single

interstitial chiasma dominate in *Enchytraeus buchholzi, E. minutus, E. lacteus, Lumbricillus bülowi, Cognettia glandulosa* and *C. sphagnetorum*. The species mentioned belong to different genera, and consequently the dominance of the two bivalent types in question must have evolved repeatedly, and independently within the different genera.

The *achiasmatic* bivalents are divided into three different types which are described on pp. 415 ff. The *Marionina subterranea-* and *Henlea perpusilla*-type are confined to two and one species respectively, their closest relatives having normal *chiasmatic* meiosis. These *achiasmatic* types are probably either not very successful or of recent origin. The third type, *Fridericia*-type, however, is found in all members of the genera *Fridericia, Buchholzia* and *Hemifridericia*, in *Enchytronia parva* and in *Achaeta bulbosa*. The *achiasmatic* bivalent is almost certainly a subsequently developed character, and the presence of three morphologically different types in three groups of species and genera which are only distantly related *inter se*, suggests a multiple origin within the family. However, the different types, considered separately, may be given some weight as a cytological criterion of evolutionary relationship. I.e., all members of the genera *Fridericia, Buchholzia* and *Hemifridericia*, which form a morphologically well defined group of genera (cf. p. 428) have bivalents of the same *achiasmatic* type, and the occurrence of the same type of bivalents in *Achaeta bulbosa* and *Enchytronia parva* may indicate that *Achaeta* and *Enchytronia* are related to the above group of genera.

To sum up, therefore, we may say — as regards the evolution of the bivalent types — that in the most primitive genus, *Mesenchytraeus*, the usual type with several chiasmata per bivalent occurs. Within other primitive genera (*Cernosvitoviella, Lumbricillus* and *Cognettia*) only chiasmata bivalents are found, except in *Lumbricillus lineatus 3x* which has *asynaptic* meiosis. Within the remaining genera *achiasmatic* bivalents have arisen independently at least three times. Similarly reductions in the number of chiasmata have taken place several times.

The large number of species with *achiasmatic* bivalents is a very characteristic feature of the Enchytraeidae, among the 71 species investigated no less than 22, or c. 31 %, have bivalents of this type. The same anomalous meiotic mechanism has been described in several other animal groups: "Higher" Diptera (Brachycera) (for references see WHITE, 1954); several Mantids and Blattids among the Orthoptera (WHITE, 1938; HUGHES-SCHRADER, 1943, 1950, 1953; SUOMALAINEN, 1946; MATTHEY, 1945 b); some Acarina (COOPER, 1939; KEYL, 1957);

a few Homoptera (SCHRADER, 1931; HUGHES-SCHRADER, 1940, 1942); and perhaps in some Mollusca (KEYL, 1955, 1956).

In the oocyte prophase and MI of Enchytraeidae with *achiasmatic* bivalents, the homologous chromosomes are not visibly divided into chromatids and there is no opening out in the centromere region until the bivalents are arranged in the metaphase plane and the centromeres are pulled apart by the developing spindle. In the dipterous fly *Apolipthisa subincana*, FAHMY (1948—49) observed a similar delay in the appearance of the equational split. This is, however, in contrast to most other cases of *achiasmatic* meiosis. Where the material is sufficiently favourable to allow a detailed analysis of the prophase, in the Mantids for example, the chromosomes are divided into chromatids already in the prophase and the centromeres separate before their orientation at the equator.

WHITE (1938) suggests that the parallel pairing without chiasmata is explicable on the basis of delay in the division of chromosomes. This may explain the situation in the Enchytraeidae. In the Mantids, however, where the chromosomes are visibly divided, the parallel pairing of late prophase involves all four chromatids of the bivalent. Another phenomenon which is common to Enchytraeids and Mantids (HUGHES-SCHRADER, 1943) may be of some importance for the evolution of *achiasmatic* bivalents, namely the prolongation of the parallel pairing of chromatids in somatic mitoses (cf. p. 416 and Fig. 111). This prolongation of parallel pairing of chromatids in mitosis may rest on the same factors that maintain the parallel pairing of homologues chromosomes in meiosis.

Since no experimental data are available it is not known whether the absence of chiasmata in Enchytraeidae means that no genetical crossing-over takes place between the chromatids of paired chromosomes. In the well-known case of the male *Drosophila* and in the males of *Phryne fenestralis* (BAUER, 1946; WOLF, 1950), the absence of chiasmata is followed by the absence of crossing-over. If this is the case in the Enchytraeidae, crossing-over is totally absent in nearly one third of the species (in the hermaphroditic Enchytraeids chiasmata are absent both in the male and female line, in all the other examples mentioned above they are only absent in one sex). If one adds to this the species where the number of chiasmata is reduced, crossing-over may be either totally absent or strongly reduced in nearly half of the species. From an evolutionary point of view it would seem very hazardous for the family, to abandon crossing-over as a means of building-up new

adaptive combinations of genes, in such a high proportion of species. However, the absence of crossing-over may be counterbalanced by the high proportion of amphimictic polyploids which just characterizes the species in question (cf. the histogram in Fig. 132 and pp. 438 and 439) since the number of free interchangeable gene blocs then is high even in the absence of crossing-over. But, of course, recombinational types do not arise.

4. Parthenogenesis

The restoration of the somatic chromosome number by fusion of second polar body and female pronucleus is the most common type among parthenogenetic Enchytraeidae and the most widespread type in parthenogenetic animals in general. Within the Oligochaeta it has, however, not been recorded before except in cases of facultative parthenogenesis in normally amphimictic earthworms (MULDAL, 1952). In other Oligochaetes (Tubificidae, CHRISTENSEN, unpubl., and earthworms, Lumbricidae, MULDAL, 1952; OMODEO, 1952), parthenogenetic reproduction is associated with a peculiar pre-meiotic doubling of the chromosome number, the number of bivalents formed being the same as that of the somatic chromosomes and two polar bodies are extruded.

Meiosis in parthenogenetically reproducing Enchytraeidae shows different deviations from normal: 1) In the two parthenogenetic *Mesenchytraeus* species meiosis is entirely normal in so far as normal eggs and spermatozoa are produced in all individuals. 2) In the parthenogenetic *Fridericia cytotypes* and *Cognettia glandulosa*, the first meiotic division in the egg is completely regular, while some individuals produce no spermatozoa due to a break down of spermatogenesis in these cases. 3) In the triploid cytotype of *Lumbricillus lineatus* no bivalents are formed and the behaviour of the chromosomes during first meiotic division excludes amphimictic reproduction, no spermatozoa are produced (CHRISTENSEN, 1960).

In *Cognettia glandulosa* and *Lumbricillus lineatus 3x* the parthenogenetic eggs must be activated by spermatozoa in order to secure normal development, in other cases spermatozoa are present but their function is unknown, and in one case spermatozoa are extremely rare. It would seem, therefore, as though the rôle of the sperm in activating parthenogenetic eggs is gradually diminishing when parthenogenesis is once established: 1) Obligatory activation of parthenogenetic eggs (*Cognettia glandulosa* and *Lumbricillus lineatus 3x*, in the latter case the spermatozoa are produced by the diploid amphimictic cytotype).

2) Spermatozoa present in the cocoons, but are perhaps without function (*Fridericia ratzeli, Mesenchytraeus pelicensis* and *M. glandulosa* may belong to this category, but there is no direct evidence). 3) No spermatozoa, and consequently no activation (*Fridericia maculata* n=45 probably belongs to this category since worms which produce spermatozoa are extremely rare, and this cytotype possess no spermathecae which could receive spermatozoa from the amphimictically reproducing n=26 cytotype, cf. *Lumbricillus lineatus*).

Obligatory activation of parthenogenetic eggs by spermatozoa has been called pseudogamy or pseudofertilization and seems to be of frequent occurrence in groups where the amphimictically reproducing forms are hermaphroditic. It has been reported from some families of Nematoda where hermaphroditism or parthenogenesis (or both) occur in some species (BÊLÂR, 1923, 1924), it is known in planarians, through the works of BENAZZI and his school (see BENAZZI, 1957), and in earthworms (OMODEO, 1952). For a general review of pseudogamy, see BENAZZI (1954).

In the Enchytraeidae, 14 out of 41 polyploid cytotypes reproduce parthenogenetically or asexually (obligatory): *Mesenchytraeus pelicensis, Lumbricillus lineatus 3x, Cognettia glandulosa, Fridericia ratzeli* n=40, 56 and 80—90?, *F. bisetosa* n=56, *F. connata* n=56, *F. maculata* n=45, *F. leydigi* n=60?, *Achaeta affinis, A. bulbosa*?, *Cognettia sphagnetorum* n=c. 54 and c. 160. In earthworms (Lumbricidae), 16 out of 24 polyploid cytotypes reproduce parthenogenetically (data from MULDAL, 1952, and OMODEO, 1952, 1955). There is, therefore, as shown in the text-table below, in both these families a strong correlation between polyploidy and parthenogenesis, compared with the situation among the diploids:

	Lumbricidae		Enchytraeidae	
	amph.	parth.	amph.	parth.
No. of dipl. cytotypes	34	1	46	1
No. of polypl. cytotypes	8	16	27	14

However, in the Enchytraeidae 27 out of 41 polyploid cytotypes still reproduce amphimictically. This ability of polyploid Enchytraeidae to reproduce amphimictically is probably due to the fact, that the bivalent types — *achiasmatic* bivalents and bivalents with only one chiasma —

which prevail among the polyploid forms, result in normal bivalent formation in polyploids, which are even multiples of the ancestral basic number. Further aspects of this topic is discussed in the next paragraph. In the earthworms the situation is reversed, since the characteristic premeiotic doubling of the chromosome number, which results in a pairing of iso-chromosomes and thereby prevents the formation of polyploids, automatically causes parthenogenetic reproduction.

Diploid parthenogenesis is divided into 1) the *ameiotic* type in which meiosis has been entirely suppressed and the maturation division(s) is mitotic and 2) the *meiotic* type in which meiosis takes place in the egg. The genetic principles which should apply in these types have been expected to be quite different. In *ameiotic* parthenogenesis segregation will not occur. Recessive mutations and structural rearrangements will tend to accumulate indefinitely in such organisms. Such forms must consequently be expected to become more and more heterozygous and eventually loose their diploid character. The entire offspring of a single female will have the same genotype as their mother except for newly arisen mutations. In *meiotic* parthenogenesis segregation may occur in the offspring of a single female if she is heterozygous. Heterozygosity will, however, be very rare in such forms since in the absence of sexual reproduction it only arises by mutations and will be eliminated or strongly reduced during each generation. Thus all organisms with meiotic parthenogenesis should tend to become homozygous for almost all loci.

Recently, however, some cases of *meiotic* parthenogenesis have been described, in which the genetic principles differ from the above description, and the sharp distinction between *ameiotic* and *meiotic* parthenogenesis from a *cytogenetic* point of view, cannot be maintained.

In Table 2 an attempt has been made to make a new division of diploid parthenogenesis from a cytogenetic point of view. More detailed discussions of the genetical principles which apply in the various types of meiotic parthenogenesis are found in SUOMALAINEN (1950) and NARBEL (1946). [+] indicates that segregation take place in the offspring of a heterozygous mother, [—] that no segregation occurs. In brackets with two sign the first indicates prereduction the second postreduction. The examples mentioned in each group are only intended to be complete for the two first groups of meiotic parthenogenesis, in the other groups only forms showing the different variations are mentioned. For a detailed list SUOMALAINEN (1950) should be consulted.

TABLE 2. *Diploid parthenogenesis and its genetical consequences.*

[+]: segregation takes place, [—]: no segregation occurs; in brackets with two signs, the first indicates prereduction, the second postreduction.

AMEIOTIC PARTHENOGENESIS. The chromosomes divide equationally at the maturation division(s). [—]

Several weevils, (SUOMALAINEN, 1947, 1949, 1954); *Pycnoscelis surinamensis*, (MATTHEY, 1945); *Bothrioplana semperi*, (REISINGER, 1940)

MEIOTIC PARTHENOGENESIS. Two nuclear divisions but only one division of the chromosomes.

No bivalents at the first maturation division. [—]

Lumbricillus lineatus 3x, (CHRISTENSEN, 1960).

Bivalents at the first maturation division.

Diploid number of bivalents at the first maturation division, two polar bodies are extruded. [—+?]

Several earthworms, (MULDAL, 1952; OMODEO, 1952, 1955); two Tubificids, (CHRISTENSEN, unpubl.); *Ptinus clavipes* f. *mobilis*, (SANDERSON, 1958; WOODROFFE, 1958).

Haploid number of bivalents at the first maturation division, the somatic chromosome number restored by fusion of:

1) First polar body and secondary oocyte. [— +/—][1]

Apterona helix, (NARBEL, 1946); *Solenobia sp.* (*lichenella*?), (NARBEL-HOFSTETTER, 1950); *Luffia ferchaultella*, (NARBEL-HOFSTETTER, 1954); *Drosophila mangabeirai*, (MURDY and CARSON, 1959); *Solenobia triquetrella*, (SEILER and SCHÄFFER, 1960).

2) Second polar body and female pronucleus. [+—]

[1] The cases, where the somatic chromosome in principle is restored by fusion of the first polar body and the secondary oocyte, is from a cytological point of view a very heterogenous group. In all cases two second metaphase plates are formed. In *Apterona*, *Solenobia* (*lichenella*) and *Luffia* no second anaphases take place, the two second metaphases fuse to form a single metaphase plate containing the somatic number of univalent chromosomes, which develops directly into the cleavage nuclei. At postreduction a heterozygous individual develops in 50 % of cases and a mosaic formed by two homozygous nuclei arises in other 50 %; details in NARBEL (1946). In the two latter species (*Drosophila mangabeirai* and *Solenobia triquetrella*) the second divisions take place, and the somatic number is restored by fusion of one haploid nucleus from each of the second anaphases, recalling the situation in *Lumbricillus lineatus 3x* (CHRISTENSEN, 1960), but in the present cases the remaining nuclei either degenerate or are extruded. At postreduction a true homozygous individual should be expected to develop in 50 % of the cases. In the *Drosophila* species, however, all flies examined are structurally heterozygous for a distal inversion, which may suppress proximal crossing-over and thus prevent postreduction; details in MURDY and CARSON, op. cit. In the Psychidae species this special mechanism probably maintains the heterozygous condition with respect to the sex chromosomes, characteristic of Lepidoptera females.

Solenobia lichenella, (SEILER, 1923); *Nemeritis canescens*, (SPEICHER, 1937); *Neuroterus baccarum*, (DODDS, 1939).[2]

3) Early cleavage nuclei, first and second polar body extruded. [++] *Solenobia triquetrella*, (SEILER, 1923, 1927; SEILER and SCHÄFFER, 1941; SEILER and GESSNER, 1950); *Trialeurodes vaporarium*, (THOMSEN, 1927).

5. Polyploidy

It appears from the text-table below that among a total of 88 cytotypes, 41 (included *Lumbricillus lineatus 3x*), or 47 %, are polyploids. It will, however, also appear that the number of polyploids within the two main types of bivalents, *chiasmatic* and *achiasmatic*, differs widely. Among 55 cytotypes with *chiasmatic* meiosis 14, or 25 %, are polyploids, while among 32 cytotypes with *achiasmatic* meiosis no less than 26, or 81 %, are polyploids:

		No. of diploid cytotypes	No. of polyploid cytotypes	Total numbers
Chiasmatic bivalents	Two or more chiasmata	36	6	42
		41	14	55
	Single chiasma	5	8	13
Achiasmatic bivalents		6	26	32
Total numbers		47	40[1]	87

[1] *Lumbricillus lineatus 3x* not included.

This must mean that *achiasmatic* bivalents favour the formation of polyploids, or at least they do not hinder their formation to such an extent as do normal *chiasmatic* bivalents. The great danger of polyploidy is the formation of multivalents at the first meiotic division resulting in a reduced fertility, and since multivalents are extremely rare in polyploids with *achiasmatic* bivalents, there must be some mechanism which prevents their formation.

In the ordinary diploid species *Callimantis antillarum*, a single large lobule in one of the testes studied was composed exclusively of tetraploid cells (HUGHES-SCHRADER, 1943). It was very characteristic of this

[2] In the latter case the first polar and secondary oocyte divide endomitotically and both nuclei later undergo segmentation divisions. No pure homozygotes are produced but only mosaics; details in SUOMALAINEN (1950).

group of cells that only tetra- and bivalents were formed, and the tetravalents consisted exclusively of metacentric chromosomes. This latter feature is not stated expressly by HUGHES-SCHRADER, but it appears from the description that all tetravalents are cross-shaped, each chromosome is paired along half its length with each of two homologues, and the four centromeres are located in the centre of the cross. There appears, therefore, to be an *arm-to-arm* pairing between the homologues in the *Callimantis* case, which rule out the formation of univalents consisting of acrocentric chromosomes, and explains the absence of tri- and univalents (this explanation would also account for the apparent excess of bivalents formed, cf. HUGHES-SCHRADER, op. cit. pp. 134—135). The same mechanism cannot, however, be the entire explanation of the absence of multivalents in most Enchytraeidae with this bivalent type; there must be some additional factor, some *chromosome-to-chromosome* pairing mechanism, which also prevents the formation of multivalents consisting of metacentric chromosomes: 1) In *Buchholzia fallax* a diploid and a tetraploid cytotype is known, the latter type is certainly autotetraploid, and in spite of the fact that several metacentric chromosomes are present, tetravalents are extremely rare. 2) In a tetraploid specimen of *Buchholzia fallax* a single octoploid egg was found in which no multivalents were formed.

From a cytological point of view, the species with *chiasmatic* bivalents are very heterogenous, and may be subdivided into the three groups mentioned on p. 403. In some of the species bivalents with only a single chiasma have become dominant (in *Enchytraeus coronatus* the majority of the bivalents have a single terminal chiasma, and in *E. buchholzi*, *E. lacteus*, *E. minutus*, *Lumbricillus bülowi*, *Cognettia glandulosa* and *C. sphagnetorum* most of the large bivalents are held together by a single interstitial chiasma), and since no multivalents can be formed in the case of chromosomes which do not form more than a single chiasma, polyploidy must be expected to occur among the above mentioned species, and actually, they comprise 13 different cytotypes and no less than 8 of these are polyploids (cf. the text-table above).

To sum up, therefore, we may say that polyploidy in the Enchytraeidae is closely associated with *achiasmatic* bivalents and bivalents with a single chiasma; this is shown schematically in the histogram in Fig. 132.

The classification of naturally occurring polyploids into the two categories autopolyploids and allopolyploids is very difficult, partly due to the fact that several intermediate conditions connect typical autoploidy

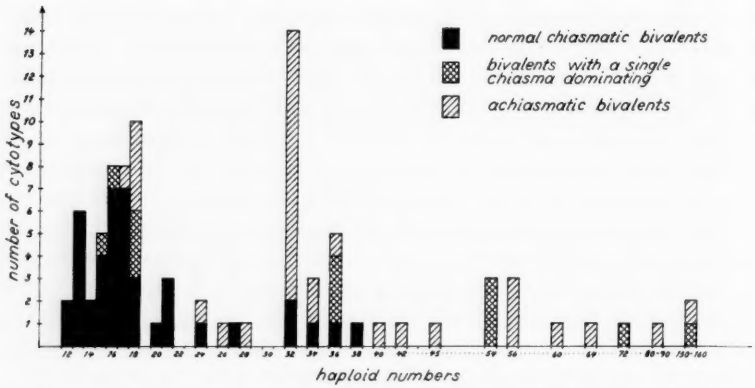

Fig. 132. Histogram showing haploid chromosome numbers and number of cytotypes in Enchytraeidae.

with typical alloploidy. In the Enchytraeidae only morphological resemblance and chromosome behaviour can be used as criteria to distinguish between the two possibilities, whereas crossing experiments and artificial production of polyploids is unfeasible at present. We have, therefore, to resign ourselves with saying that autoploidy is most likely in cases where completely identical cytotypes, with chromosome numbers which are multiples of the same basic number, are known within the same species. Conversely, in cases where a polyploid is described as a separate species, morphologically distinguishable from related diploid species, alloploidy may be at hand, but the occurrence of a diploid cytotype which is either extinct or not found yet cannot be excluded. Although these criteria are unsatisfactory, it seems justified to assume that autoploidy is at hand when the cytotypes in question are morphologically completely identical. This view is supported by the occurrence of the different cytotypes of heterogenous species. Although the cytotypes are found within the same geographical area, they rarely occur together in the same habitat. Only in four species have different cytotypes been found in the same locality: *Lumbricillus lineatus* (CHRISTENSEN, 1960, and CHRISTENSEN and O'CONNOR, 1958), *Fridericia maculata* (the n=45 cytotype possess no spermathecae and worms with mature spermatozoa are extremely rare), *Fridericia ratzeli* n=40 and 56 (both cytotypes reproduce parthenogenetically) and *Henlea perpusilla.*

Based upon these criteria, 12 polyploids, which are multiples of a morphologically identical cytotype, are autoploids (*Cognettia sphagne-*

torum n=c. 160, *Lumbricillus lineatus 3x*, *Enchytraeus buchholzi* n=36, *E. lacteus* n=36, c. 54 and 72, *E. coronatus* n=36, *Fridericia connata* n=64, *Buchholzia fallax* n=36, *Hemifridericia parva* n=28 and c. 42, *Henlea perpusilla* n=c. 34), and 7 cases where the origin is less obvious, may also belong to this category (*Fridericia maculata* n=45, *F. bisetosa* n=56, *F. connata* n=56, *F. leydigi* n=60 and *F. ratzeli* n=40, 56 and 80—90); whereas 8 species may be alloploids (*Mesenchytraeus pelicensis*, *Cognettia glandulosa*, *Achaeta bohemica*, *A. affinis*, *A. danica*, *A. bulbosa*, *Henlea jutlandica* and *Marionina clavata*). The tetraploid *Fridericia* species are not included in the latter category because the speciation within this genus has probably taken place at this level (cf. p. 396). Autoploidy seems, therefore, to be of greatest importance in formation of polyploid Enchytraeidae.

An interesting cytological difference exists between the two categories of polyploids mentioned above, and this may, after all, turn out to be the best criterion to distinguish between them. Among those which are likely to be autoploids, meiosis differs from normal in *all* cases: in *Lumbricillus lineatus 3x* no bivalents are formed, in *Enchytraeus buchholzi*, *E. lacteus*, *E. coronatus* and *Cognettia sphagnetorum* the bivalents have only one chiasma each, in *Buchholzia fallax*, *Henlea perpusilla*, *Hemifridericia parva* and the *Fridericia* cytotypes mentioned the bivalents are of the *achiasma-type*. In the other category — the alloploids — on the contrary, meiosis is apparently normal except in two cases: *Achaeta bulbosa* and *Cognettia glandulosa* (in the former the bivalents are of the *achiasma*-type, on the latter the homologues are held together by a single chiasma). This difference between the two categories of polyploids is certainly more than a coincidence since the abnormal meiotic mechanisms mentioned all hinder the formation of multivalents and thus make autoploidy possible, whereas polyploid forms with normal *chiasmatic* meiosis have to be alloploids in order to avoid formation of multivalents.

In the higher plants it is well known that polyploidy has been a major factor in evolution; it is in fact possible that more than half the species of higher plants are of a polyploid nature. Compared with that, polyploidy is extremely rare in the animal kingdom (WHITE, 1954; BUNGENBERG DE JONG, 1957).

The first suggestion to account for the rarity of polyploidy in animals compared with plants was that of MULLER (1925). He pointed out two striking differences in the evolutionary pattern of plants and animals, namely the scarcity of dioecious species and the widespread occurrence

of polyploidy in plants, against the scarcity of polyploids in animals where most species are dioecious. He linked these two phenomena together and explained the absence of polyploidy among animals through their dioecious outbreeding system, and supported his argumentation by the *Drosophila* type of sex determination, which would inevitably break down in case of polyploidy. According to MULLER's view polyploidy should be effectively confined to those animal groups in which reproduction is hermaphroditic, parthenogenetic or vegetative, where the number of polyploids should be expected to be just as high as in plants.

In the hermaphroditic animals, however, the evidence is somewhat conflicting. In some hermaphroditic groups polyploidy is extremely rare. In pulmonate snails the chromosome number is known of nearly 100 species and only one polyploid has been reported, *Gyraulus circumstratus* (BURCH, 1960). Among 15 tapeworms there was no evidence of polyploidy (JONES, 1945), and data of BRITT (1947) on 49 species of digenic trematodes provide no support for the occurrence of polyploidy. In leeches there are a few possible polyploids, but the evidence is not conclusive (WENDROWSKY, 1928). In other hermaphroditic groups, however, polyploidy is of wide occurrence, i.e. planarians (JONES, 1944; AEPPLI, 1952; BENAZZI, 1945, 1949; LEPORI, 1950; DAHM, 1958); and oligochaetes (MULDAL, 1952; OMODEO, 1952, 1955).

MULDAL (loc. cit.) and OMODEO (1952) seem to join MULLER's conception that polyploidy amongst the earthworms occur as readily as amongst plants. But both these authors overlook, that according to MULLER's hypothesis one should expect amphimictically reproducing polyploids, and the majority of polyploid earthworms are actually parthenogenetic. In the Enchytraeidae the evidence may be in accordance with MULLER's expectation, 41 polyploid cytotypes are known, and 27 are cross-breeding. A closer examination of meiosis in these amphimictic forms shows, however, that the bivalent formation is abnormal (only one chiasma or *achiasmatic*) except in four cases (*Henlea jutlandica, Achaeta danica, A. bohemica* and *Marionina clavata*, cf. p. 406). This means that less than 5 % of 88 cytotypes investigated are amphimictic polyploids with completely normal *chiasmatic* meiosis. In the earthworms 8 out of 59 cytotypes, or less than 14 %, are amphimictic polyploids.

The number of amphimictic polyploids with normal meiosis is, therefore, higher in these two hermaphroditic families than among dioecious animals in general. This may be explained on the basis of MULLER's

hypothesis; on the other hand, the number of amphimictic polyploids with normal meiosis is far below the number found in higher plants, there must, therefore, be other factors than those pointed out by MULLER which make the formation of polyploids more difficult in animals than in plants.

WETTSTEIN (1927) and STEBBINS (1950) believe that one reason why polyploidy is so much rarer in animals than in plants is that the developmental processes of cellular differentiation are much more complicated in animals, and hence more liable to be disturbed by polyploidy. This explanation probably has some validity, as far as qualitative modifications are concerned, whereas the high number of autoploid Enchytraeidae suggest that too much importance should not be attached to it when only quantitative modifications take place.

Another (perhaps supplementary) explanation may be that gametes from different species stand a greater chance of meeting in plants than in animals because the majority of the latter have internal fertilization. Consequently one might expect polyploidy to occur in animals with external fertilization, i.e. mostly in marine animals. The available chromosome data are too meagre to support this hypothesis, but polyploidy is not unlikely to occur among echinoderms (see MAKINO, 1951).

Both the WETTSTEIN-STEBBINS explanation and the explanation put forward here involve that the classical scheme for the formation alloploids through hybridization (WINGE, 1917) is unlikely to take place in animals, and one must, consequently expect the majority of the actual known polyploids to be autoploids. It has already been shown (p. 441) that the situation in the Enchytraeidae is in accordance with this expectation. In these cases autoploidy is considered most likely when the polyploid in question is morphologically identical with another cytotype whose chromosome number is a lower multiple of the same basic number. If this criterion is used in general, many polyploid animals are likely to be autoploids, and the majority of the remaining polyploids are known from animal groups where autoploidy is of wide occurrence: several planarians (see DAHM, 1958, for review); several earthworms (MULDAL, 1952; OMODEO, 1952, 1955); several weevils (see SUOMALAINEN, 1954, for review); *Artemia salina* (BARRIGOZZI, 1946; GOLDSCHMIDT, 1952); *Trichoniscus elizabethae* (VANDEL, 1934); *Solenobia triquetrella* (SEILER, 1923, 1927; SEILER and SCHÄFFER, 1941); *S. lichenella* (SEILER, 1923; NARBEL-HOFSTETTER, 1950); *Ptinus clavipes* f. *mobilis* (SANDERSON, 1958); *Potamopürgus jenkinsi* (RHEIN, 1935; SANDERSON, 1940).

The meiotic mechanism must, consequently, be expected to be aber-

rant in these cases, and actually, only the Enchytraeidae and the *Solenobia* cytotypes form the haploid number of bivalents at the first meiotic division. The special bivalent types, which makes this possible in the Enchytraeidae, are described above, and in Lepidoptera females the bivalents are apparently held together by a single terminal chiasma (SUOMALAINEN, 1953).

The occurrence of polyploidy in the animal kingdom may, therefore, tentatively be summarized as follows: 1) An X/autosome sex determining mechanism effectively prevents the formation of polyploids. 2) An Y-mechanism and hermaphroditism does not automatically prepare the way for polyploidy, because alloploids rarely arise, due to genetical incompatibility between genomes from different species or because these rarely meet, and only alloploidy is consistent with normal bivalent formation. 3) Polyploidy, therefore, only occurs to any appreciable extent when the maturation divisions are so abnormal — no bivalents, pre-meiotic doubling, one chiasma per bivalent, *achiasmatic* bivalents (in Enchytraeidae) — that no multivalents are formed in autoploids. 4) The close connection between parthenogenesis and polyploidy in animals is, on one hand, due to the fact that some of these abnormal meiotic types in themselves involve parthenogenetic reproduction, on the other hand, when once established, parthenogenesis facilitates the formation of polyploids.

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SUMMARY

Chromosome numbers are reported from 71 different species, comprising 88 different cytotypes. There are maxima of chromosome numbers, at n=12—21, at even numbers from 32—36 and again at n=54 and 56 (cf. Table 1 and Fig. 132).

Several cases of both intra- and interspecific polyploidy are recorded (cf. p. 398); 41 cytotypes, or 47 %, are of a polyploid nature.

Three kinds of reproduction have been found: 1) Amphimixis (p. 399); 2) Parthenogenesis (associated with pseudo-fertilization) (p. 399); 3) Asexual reproduction (either obligatory or co-existing with sexual reproduction) (p. 402).

Three main types of meiosis are described: 1) *Chiasma-type*, p. 403; 2) *Achiasma-type*, p. 415; 3) *Asynapsis-type*, p. 425. The two former types are again subdivided into three groups each, and descriptions of meiosis are given in relation to bivalent types and reproductive mechanisms of diploid and polyploid forms; pp. 406, 408, 411 and pp. 418, 422 and 424 respectively.

It is concluded, that n=16 is the ancestral haploid number for the family, and both increase and decrease in chromosome number have taken place at this original haploid level (p. 430).

The evolution of the different bivalent types is discussed on pp. 431 ff. Bivalents with several chiasmata each is the ancestral type within the family, but reductions in the number of chiasmata have taken place several times. Similarly *achiasmatic* bivalents have arisen independently at least three times, and there seems to be a *chromosome-to-chromosome* pairing between the homologues of this type (p. 439).

It is shown that the occurrence of polyploidy is closely associated with the *achiasma-type* of bivalents (p. 438) and *chiasmatic* bivalents with a single chiasma (p. 439 and the histogram in Fig. 132).

14 out of 41 polyploid cytotypes reproduce parthenogenetically or asexually (p. 435), the remaining 27 are cross-breeding. This is contrast to the situation elsewhere in the animal kingdom where polyploidy is more closely associated with parthenogenetic reproduction. The high number of amphimictic polyploids among the Enchytraeidae is explained through the occurrence of *achiasmatic* bivalents and bivalents with a single chiasma, which prevent the formation of multivalents, and hence make amphimictic reproduction possible in polyploids.

The genetical consequences of diploid parthenogenesis is briefly discussed, p. 436, and summarized in Table 2.

It is likely that autoploidy has been of greatest importance in formation of polyploid Enchytraeidae (p. 439) and it is shown that among the autoploids meiosis differs from normal in all cases, and these abnormal meiotic mechanisms all hinder the formation of multivalents (p. 441). Among the alloploids meiosis is apparently normal except in two cases; 8 species may be alloploids, arisen through hybridization (p. 441).

The occurrence of polyploidy in the animal kingdom is discussed, and it is concluded, that only where the meiotic and reproductive patterns allow the formation of autoploids, does polyploids occur in any numbers (p. 58).

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CYTO-ECOLOGIC PROBLEMS IN NORWEGIAN FLORA GROUPS

DISTRIBUTION AND SIGNIFICANCE OF POLYPLOIDY[1]

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I. INTRODUCTION

IN 1876 the late AXEL BLYTT, Professor at the University of Oslo, published a paper which created great interest. Its title was: "Essay on the immigration of the Norwegian flora during alternating rainy and dry periods." Norway, with its long coast and mountains occupying the interior, has a highly variable topography. The mountain ridges follow a direction parallel to the coast, and owing to the different degrees of influence of the sea, the climate varies very considerably from one part of the country to the other. The fjord landscapes in Western Norway have a pronounced oceanic climate with cool summers, mild winters, and a high rainfall, evenly distributed throughout the year. The valley landscapes in the "rain shadow" in Eastern Norway have warm and dry summers and cold winters with snow lying for several months. The climate in the mountains of Southern Norway with peaks up to 2,468 m. resembles that of the areas north of the Arctic Circle, in the counties of Nordland, Troms and Finmark.

The composition of the flora in the different climatic areas differs greatly. BLYTT separated four groups of lowland plants, climatically conditioned, which he named after the post-glacial periods in which he thought they had immigrated to Norway: the Boreal, the Atlantic, the Sub-Boreal and the Sub-Atlantic groups. The Boreal and Sub-Boreal groups are today found only in Eastern Norway, the first as far west

[1] The present work was delivered as a lecture on a topic, chosen by the author, at her disputation for the degree of Doctor of Philosophy at the University of Oslo, February 1960.

as in the inner fjord districts of Western Norway and Trøndelag, the latter only in the south-eastern part of Eastern Norway. The Atlantic and the Sub-Atlantic groups are found only along the coasts in Western Norway with somewhat varying boundaries to the east and north. Today, we know much more about the migratory history of Norwegian flora after the Ice Age, than did BLYTT, thanks to the work of our pollen analysts. Although now out of date BLYTT's theories still contain a considerable amount of truth and are therefore referred to when phyto-geographical problems are discussed.

On reading the discussions of the varying significance of polyploidy in European and Arctic floras, the present author found it of interest to compare the frequency of polyploids in the different flora groups in Norway against the background of the migratory history of the flora after the retreat of the ice sheet, which covered most of the country in the last Ice Age, i.e. about 30,000 years ago.

II. OCCURRENCE OF POLYPLOIDY

The modern trend of phyto-geography, which can also be called cyto-ecology, is to investigate the variations and distributions of the higher plants in nature on the basis of the chromosomes, their numbers and structures. It can be dated back to the year 1917 when ØIVIND WINGE formulated his hypothesis of hybridization followed by polyploidy as a means of species formation in nature. Polyploid species are genetically related species with chromosome numbers forming multiple series with a common basic number. WINGE's hypothesis has been verified by analyses of polyploid complexes of natural species and of crop plants, and further, experimentally, by synthesis of allopolyploids. Today we know from thousands of counts of chromosome numbers that polyploidy is widespread among the higher plants, though very irregularly distributed. It has had no significance in the evolution of new species in the Gymnospermae, but occurs frequently in a variety of groups within all categories of the Pteridophyta and in the Angiospermae. In the Pteridophyta it seems that polyploidy has reached a much higher level than in any other group of plants. This is proved by the high chromosome numbers of the most different polyploid complexes.

It seems probable that, with increasing age, genera with high chromosome numbers arise. High chromosome numbers have, therefore, been regarded as a sign of antiquity in a genus. This is particularly true of polyploids, which, having outlived their low-numbered ancestors, are

regarded as antique and as representing the last links of highly developed polyploid species. Many categories of the Pteridophyta can be mentioned as examples of such old polyploids. *Psilotum* (diploid with 2n=c. 100, tetraploid with 2n=c. 200) and *Tmesipteris* with 2n=over 400 are regarded as high-polyploid survivors of the primitive Psilotales. In *Equisetum* all species have 2n=216, and *Ophioglossum vulgatum* has 2n=500 odd (MANTON, 1950).

Genera of modern ferns, however, in spite of their high chromosome numbers, contain, like the Angiospermae of today, species with several related chromosome numbers. Especially in the *Polypodiaceae*, as studied by MANTON in Great Britain, there exist genera rich in species with polyploid chromosome numbers dispersed throughout the flora in a variety of environments. They give the general impression, says IRENE MANTON, of having resulted from a recent wave of polyploidy which has affected the flora as a whole and led to a partial replacement of the low-numbered species by their high-numbered descendants, a process which, she thinks, is perhaps still continuing.

The chromosome numbers are generally much lower in the Angiospermae than in the ferns. They are most often lower than 50. They are seldom as high as 100, depending on the degree of polyploidy in the genus. This agrees with the younger age of the Angiospermae, the youngest of the three large groups of higher plants. The polyploid Angiosperm genera contain, typically, one or more polyploid species in contrasting environments. Polyploidy, however, is very irregularly distributed among genera and families, and the phenomenon seems to have been of varying significance in different geographical areas. Of this, STEBBINS (1950) has given a survey.

On an average, the highest percentages of polyploids are found in perennial herbs, a smaller percentage in annuals, and the lowest percentage in woody plants. The following genera are almost completely devoid of polyploidy: *Carpinus*, *Corylus*, *Castanea*, *Fagus*, *Quercus* (28 diploid species, 1 tetraploid, the latter in Japan), *Ulmus*, *Ficus*, *Morus*, *Ilex*, which all belong to temperate regions. Arctic and alpine areas have few woody plants, and the following genera that occur represent polyploidy: *Betula*, *Alnus*, *Sorbus*, *Salix*. Moreover, most of the dwarf scrubs dominating in these areas belong to polyploid genera.

There are also many variable genera of perennial herbs completely devoid of polyploidy. *Epilobium* can be mentioned as a good example of a Norwegian genus with many species, which, without any change in chromosome numbers, have adopted themselves to contrasting en-

vironments. Its 11 species, all with 2n=36 chromosomes, have occupied the whole country, from sea level to the highest mountains; from south to north, a distance covering more than 13 degrees of latitude, that is, from temperate regions to the Arctic; and, further, from the moist oceanic climate in the west, to the warm dry valleys in the east, in all kinds of environments. *Umbelliferae* can be quoted as an example of a family with a small degree of polyploidy in all geographical areas.

III. SIGNIFICANCE OF POLYPLOIDY IN EUROPEAN FLORAS

The cause of the apparently varying significance of polyploidy in different geographical areas has been vigorously discussed in our time. The varying occurrence in European floras was debated first for the obvious reason that it was these floras which are best known as to chromosome numbers. TISCHLER, supported by LÖVE, has tried to explain that there is an increase in the number of polyploid species from south to north in Europe, and that arctic areas and mountain floras have a relatively high content of polyploids, compared with that of lowland floras.

To demonstrate some of this variation a few of the percentages of polyploids found in various floras from TISCHLER and LÖVE's comparisons have been listed in Table 1. They show that the proportion of polyploid species in Europe increases northwards in the following se-

TABLE 1. *Contents of diploids and polyploids in European floras (Angiospermae).*

After TISCHLER, 1955.

	Diploids	Polyploids	D + P	N	%
Cyclades	365 = 63 %	214 = 37 %	56	1186	53.5
Hungary	754 = 51.4	714 = 48.6	136	2039	78.7
Rumania	1117 = 53.2	982 = 46.8	159	3365	67.1
Central Europe	1038 = 49.1	1076 = 50.9	210	2909	79.9
Schleswig-Holstein	438 = 45.5	525 = 54.5	79	1081	96.4
Great Britain	671 = 46.7	765 = 53.3	113	1778	87.1
Sweden	568 = 43.1	753 = 56.9	106	1526	93.5

D+P=Number of species with intra-specific polyploidy
N=Number of species in the flora
%=Percentage of species known as to chromosome numbers

TABLE 2. *Contents of diploids and polyploids in arctic floras (Angiospermae).*

	Dicotyledones		Monocotyledones		D + P	N	%
	Dipl.	Polypl.	Dipl.	Polypl.			
Iceland TISCHLER, 1955	149	196	22	135		502	100
L. & L., 1956	=43.2 %	=56.8 %	=14 %	=86 %			
Greenland	89	117	14	70	46	437	69
J., S. & W., 1958	=43.24 %	=56.8 %	=16.7 %	=83.3 %			
Pearyland	13	23	3	25		64	100
HOLMEN, 1951	=56.5 %	=43.5 %	=12 %	=88 %			
West Spitzbergen	28	52	1	41		135	82.1
FLOVIK, 1940	=35 %	=65 %	= 2.4 %	=97.6 %			

D+P=Number of species with intra-specific polyploidy
N=Number of species in the flora
%=Percentage of species known as to chromosome numbers from within the area

quence: the Cyclades, Hungary, Rumania, Central Europe, Schleswig-Holstein, England, Sweden from 33.7 % in the south to 56.9 % in the north. In the Arctic, the percentages are high in all areas. In Iceland, and Greenland as a whole, there are 69.3 % polyploids in each country (LÖVE and LÖVE, 1956; JØRGENSEN, SØRENSEN and WESTERGAARD, 1956). The numbers are higher in other, more restricted areas. In Pearyland in northernmost Greenland there are 81.5 % polyploids (HOLMEN, 1951), in West Spitzbergen 73.6 % (FLOVIK, 1940).

In Table 2 is listed the distribution of diploids and polyploids in the Monocotyledones and Dicotyledones of these latter areas. As to the arctic floras, it must be stressed that percentages mentioned above are only those calculated from chromosome numbers determined on material from within the areas. Besides the Greenland flora there is only one arctic, extra-European flora which has been investigated as to chromosome numbers, viz., the one from Kolguyev Island east of Cape Kanin, where there are 64 % polyploids according to SOKOLOVSKAJA and STRELKOVA (1941).

Exception has been taken to the percentages of polyploidy having been calculated for the European floras even if the chromosome numbers have not been determined on material from within the areas. It is only TISCHLER's investigations of the chromosome numbers of the flora of Schleswig-Holstein which have been based solely on material from

within the area. TISCHLER considered all species within the areas, with the exception of the microspecies of *Rubus*. JØRGENSEN, SØRENSEN and WESTERGAARD omitted the *Cyperaceae*. Even if the aneuploid numbers of the genera originate through modified forms of polyploidy, it is difficult to separate the diploid and polyploid numbers. Species which show intra-specific polyploidy are omitted in all cases as we do not know the geographical distribution of their diploid and polyploid numbers. There are surprisingly many species showing intra-specific polyploidy. In the flora of Central Europe these amount to 8.0 % of the species.

The principal theories formulated to explain the varying significance of polyploidy in the different floras are as follows. TISCHLER, supported by Á. and D. LÖVE, claims, in accordance with HAGERUP's original hypothesis that polyploid species are more tolerant of extreme ecological conditions than their diploid relations. They attribute the higher percentages of polyploids in northern areas and in the mountains to the great hardiness of the polyploids. They maintain that the polyploids are more resistant to severe cold, for instance, than the diploids. Á. and D. LÖVE explain the high proportion of polyploids in arctic areas in the same manner. They consider that the polyploids must have been more resistant than the diploids during the glaciations, and that for this reason more of the polyploids survived the Ice Age than did their low-numbered relations.

These theories have been criticized by many authors. It has been stressed that a complete change to new and severe conditions cannot possibly result from a mere increase in chromosome sets. It seems as though we cannot give a general explanation of the phenomenon, and that each flora has to be analysed as to its content of species. It has, for example, been experimentally shown that frost resistance of related species is not correlated to differences in chromosome numbers (BOWDEN, 1940; CLAUSEN, KECK and HIESEY, 1945).

GUSTAFSSON (1948) has reported that certain ecologic plant groups have a greater number of polyploid species than others, this being connected with certain life forms disposing of polyploidy. As mentioned above, annuals of a complex are most often diploid, whereas, on an average, high percentages of polyploids are found in perennial herbs, and especially among those having a strong vegetative propagation (cf. also MÜNTZING, 1936).

The Mediterranean floras are characterized by many diploid annuals and by woody genera in which the faculty of polyploidy is absent. The

flora in Sicily has about 30 % polyploids, the same low percentage as in the Cyclades (TISCHLER, 1935). In the Arctic and in the mountain areas in Northern Europe, there are few annuals. There is a single annual in the Norwegian mountains, *Koenigia islandica*, which is tetraploid. These areas are dominated by grasses, or by grassy plants of the families *Juncaceae* and *Cyperaceae*. A large percentage of the grasses in the Arctic are perennials, with strong vegetative propagation by runners, vivipary or apomictic seed formation, a life form which almost always is connected with polyploidy. "Grasses" as a collective group can comprise more than 1/3 of all plant species in arctic areas. On Scandinavian mountains a grass region exists at greater heights than the region with dwarf scrubs, *Betula nana* and mountain willows. More than 90 % of these grasses are polyploids, which involves a rise in the percentage of polyploids in the area.

STEBBINS claims that the high percentage of polyploidy in arctic and alpine areas is, in the main, dependent on the dominance of grasses in the vegetation cover of these areas. A fact supporting this view can be mentioned here. According to TISCHLER's "Chromosome Atlas" the grasses only amount to 14 % of the inventory of lowland species in Central Europe, and of these many are diploids. But the problem is not as simple as it might appear. If we analyse the floras along other lines, analysing their inventory of polyploid genera on, for instance, the basis of historical facts it can be proved that other factors must be taken into account. Or again, the question of how to explain that 56.8 % of the Greenland Dicotyledones are polyploid, is still unanswered. Originally, the intention of JØRGENSEN, SØRENSEN and WESTERGAARD in their cytological studies of the Greenland flora was to contribute to the general discussion of arctic polyploidy, but this they refrained from doing, chiefly because so few chromosome numbers are known from arctic America and Siberia.

IV. POLYPLOIDY IN NORWEGIAN FLORA GROUPS

To procure a preliminary orientation in the distribution of polyploid species in Norway the present author has ascertained the percentages of polyploids in the different climatically conditioned flora groups in this country (Tables 3 and 4). But she did not think it of interest to consider the genera which have hitherto been known to include almost only polyploids. Therefore, the Pteridophyta and the apomicts belonging to the family *Rosaceae* and to the genera *Taraxacum* and *Hieracium*

TABLE 3. *Grouping of the Norwegian flora.*

Groups	Distribution
Larger groups:	
1. Lowland	Lowland plants occurring from sea level up into the coniferous forest belt.
2. Mountains	Mountain plants occurring in the sub-alpine and alpine regions. Ubiquitous or disjunct distribution.
3. Whole country	Over the whole country, from sea level and up among the mountains.
Narrower groups:	
4. Bi- or unicentric species	Mountain plants with disjunct distribution.
Lowland plants:	
5. Nordland	Occurring in Southern Norway with northern limit in Nordland. 66° N. Lat.
6. Trøndelag	Occurring in Southern Norway with northern limit in Trøndelag. 64° N. Lat.
7. S. of Dovre Mts.	Occurring in Southern Norway, but seldom north of the Dovre mountains. 62° N. Lat.
8. Boreal group (BLYTT)	South-Eastern Norway and inner fjord districts in Western Norway and Trøndelag.
9. Sub-Boreal group (BLYTT)	Only in South-Eastern Norway.
10. Southern species	Occurring in southernmost Norway, south of the Oslo Fjord. 60° N. Lat.
Coastal plants:	
11. FÆGRI's group	Coastal plants in Norway.
12. Oceanic spp.	Oceanic species also farther south in Europe.
13. *Bromus Benekenii* group	Oceanic accentuated, but somewhat restricted in distribution.
14. Shore plants	Halophilous or shore plants.

have been omitted. Further, all apomictic grasses and the *Cyperaceae* have also been omitted.

However, we must fully realize the hazards involved in finding the percentages of polyploids also in other Norwegian plant groups, since almost all chromosome numbers have been determined on material from outside Norway. The latest cytological investigations from Central Europe and England have, namely, revealed that a large number of the most common species show intra-specific polyploidy. All species show-

TABLE 4. *Content of diploids and polyploids in Norwegian flora groups.*

Groups	Diploid		Polyploid		D + P Nnmber of species	Chr. numb. unknown Numb. of species	Weeds Number of species		
	Number of species	%	Number of species	%			D	P	D + P
Larger groups:									
1. Lowland	245	77.3	72	22.7	41	17	Weeds omitted		
2. Mountains	96	65.8	50	34.3	7	9			
3. Whole country	67	53.2	59	46.8	9	—			
Narrower groups:									
4. Bi- or unicentric spp.	36	50.0	36	50.0	3	7	0	0	0
Lowland:									
5. Nordland	26	63.4	15	36.6	17	2	8	2	3
6. Trøndelag	29	80.6	7	19.4	11	3	4	4	3
7. South of Dovre Mts.	23	76.7	7	23.3	3	2	3	1	1
8. Boreal group	39	90.7	4	9.3	6	4	2	3	0
9. Sub-Boreal group	42	93.3	3	6.7	8	3	2	2	0
10. Southern spp.	18	65.0	10	35.0	4	3	0	0	1
Coastal plants:									
11. FÆGRI's group	69	75.0	23	25.0	13	4	Weeds omitted		
12. Oceanic spp.	23	57.5	17	42.5	2	3			
13. *B. Benekenii* group	15	78.0	4	22.0	1	1			
14. Shore plants	26	57.8	19	42.2	1	2			

D=Diploids, P=Polyploids, D+P=species with intra-specific polyploidy.

ing intra-specific polyploidy outside Norway, but which have not been investigated as to chromosome conditions with us, have been omitted in the calculations as we do not know which number, diploid or polyploid, occurs here. However, some uncertainty is still attached to the percentages found, since we do not definitely know whether the remaining species comprise one chromosome race only.

As a matter of course, the percentages found by the present author are not directly comparable with the ones determined from other geographical regions, as these are based on another selection of the species concerned.

Discussing the occurrence of polyploidy in the different climatic areas in Norway on the basis of the percentages listed in Table 4, even if some uncertainty is attached to the numbers, the present author has

come to conclusions that bring to light new points of view, which may be of interest.

Tables 3 and 4 contain two different groupings of our flora. Three larger and 11 narrower groups have been considered. The three larger groups are set up against each other according to the vertical distribution in Norway of the species included, irrespective of their total area. One of these, group 1, includes all lowland species growing from sea level up into the coniferous forest belt, some of them from south to north, but none ascending into the mountain birch belt. Another group (2) includes species occurring in the mountain birch woods or in the tree bare, alpine region, as well as in the arctic north. There is 34 % polyploids in this wide group of arctic-alpine species, that is, more polyploids than in the group (1), comprising the lowland plants, which has 22.71 % polyploids. The percentage of polyploids is again higher (46.8 %) in the group (2) of species growing over the whole country at all heights, from south to north.

Below, we will chiefly consider the narrower groups of species with more restricted boundaries.

1. The arctic-alpine flora groups

The percentages of polyploids differ in the two groups of arctic-alpine species, considered. The larger group 2, mentioned above, including all our arctic-alpine species, both ubiquists occurring throughout the whole mountain range, from south northwards to Finmark, as well as the species with disjunct distribution, has a lower number of polyploids than the narrower group 4, 34 % as against 50 % in the latter. The narrow group contains only our rare species, viz. the ones which are said to have bicentric or unicentric distribution because their stations fall within two separate areas, one, smaller, in the mountains in Southern Norway, the other, larger in extension, north of the Arctic Circle (Fig. 1).

As is well known it is especially the latter group of species, with disjunct distribution, which has been discussed in connection with the theory of glacial survival in ice-free areas in Norway, either along the Møre coast outside the southern inland "island" of rare species, or at different places along the coast north of the Arctic Circle. If the theory of glacial survival is correct, this group of rare arctic-alpine species represents the most ancient flora element in Scandinavia.

The polyploids amount to 50 % in the group (4) of rare mountain

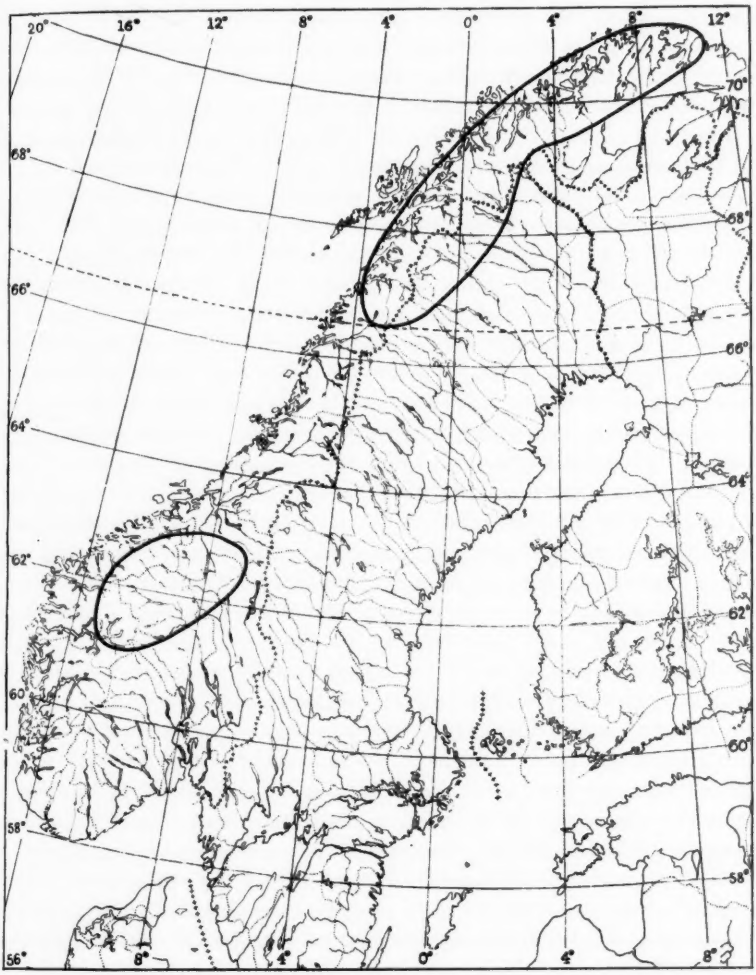

Fig. 1. Map of the two island areas in Scandinavia curiously rich in mountain species.

species, which is a high percentage considering how the percentages were computed, and the question is in what manner this can be explained.

We must make comparisons with other alpine floras. In the course of the last 10 years many polyploid complexes belonging to the alpine

flora of Central and Southern Europe have been investigated from a cyto-ecologic point of view, and many interesting facts have been revealed. During the Quaternary period the ice sheet covered the mountains of Central and Southern Europe. However, it has been proved that in the peripheric Alps, which lay within the border of the ice sheet but were less glaciated than the high, central Alps, there grow today many species with restricted ecology and distribution; and the interesting point is that they are diploid. The origin of these species has been discussed. They have been considered as ancient types, with roots going directly back into the Tertiary flora of the Alps. It is thought that this Tertiary alpine flora must have developed from the local flora when conditions changed at the end of the Tertiary period, adapting themselves to the climate of greater heights, without any change in chromosome numbers. It is also supposed that the lowland types, on the plains, became extinct during the glaciations, whereas the Tertiary alpine types survived on nunataks, and have moved very little since the ice melted (cf. FAVARGER, 1958).

The occurrence of polyploids in the flora of the Alps as a whole has been discussed in relation to arctic polyploidy. The percentage of polyploids amounts to 56.2 in the Alps, a number which is much lower than the ones found in arctic floras (Table 2). The former number has been calculated in the same way as the latter, all genera being taken into consideration. FAVARGER maintains that the reason why the percentage of polyploids is lower in the Alps than in the Arctic is chiefly because the ancient element of Tertiary plants includes so many diploids which are missing in the north. He considers that the high number of these diploids lowers the percentage of polyploids in the Alps. Both FAVARGER and MERXMÜLLER emphasize that the central high Alps, which were more glaciated than the peripheric Alps, comprise a higher proportion of polyploids than the lower, peripheric Alps. They discuss in that connection LÖVE's theory of arctic polyploidy. As mentioned above, this theory suggests that the polyploids could stand the hard conditions during the Quaternary glaciations better than the diploids, and that, therefore, more polyploids than diploids survived the Ice Age.

The investigations — now in progress in Switzerland and Austria (FAVARGER and his collaborators, EHRENDORFER) — of the detailed geographical distribution of the varying chromosome races of species showing intra-specific polyploidy in the Alps and the surrounding lowland will certainly clear up many problems concerning the recent

history of the flora of the Alps. The number of species with varying chromosome numbers have appeared to be surprisingly high, also in species in which the morphological differentiation is not pronounced, even if the ecological variation is clear, the chromosome races belonging to different milieus or climatic areas.

The large content of polyploids in the High Alps is not first and foremost attributed to the strong resistance of polyploids to severe conditions, but rather to the longer distances the plants have traversed before reaching the High Alps. With MERXMÜLLER we can say that a "Sippengliederung" has taken place contemporaneously with an "Arealbildung". It seems as though the diploid hibernated species have been the progenitors of new polyploids, which occupied the areas laid bare after the melting of the ice.

FAVARGER points out that many of the immigrants to the Alps which originally came from the Himalayas and other Asiatic mountain ranges belong to the polyploid element in the Alps. As example he mentions species of the genera *Androsacae*, *Primula* and *Gentiana*. During the wanderings of plants over smaller or greater distances the ecologic and climatic conditions change, and miscellaneous populations arise. All these new conditions offer chances for new polyploids to appear and to establish themselves in the floras.

SOKOLOVSKAJA and STRELKOVA's analyses of the alpine flora in the Near East coincide with these points of view. Especially is this true of the flora of the Caucasian mountains. Here they found a percentage of polyploids amounting to 50 only, calculated according to the old methods, all genera being taken into consideration. The authors explain this relatively low occurrence of polyploids in accordance with FAVARGER. The last elevation of the mountains in Caucasia, to twice the height, took place during the Quaternary period, and they maintain that a large part of the Caucasian alpine flora of today originates directly from the local Tertiary flora at lower heights. This local flora moved with the elevation of the mountains, and many species adjusted themselves to life at greater heights. The Russian authors think that these species represent the large number of diploid species belonging to the alpine flora of today, whereas most of the species which wandered long distances to reach Caucasia are polyploid. It may be mentioned in this connection that SKOTTSBERG (1939), similarly connects the high percentage of polyploids in the Hawaiian Islands with the long migratory distances. Many species in these islands originate from antarctic areas.

Generally, we must think that isolated, polyploid species with high chromosome numbers, which grow far removed from the area where several of their ancestors with lower chromosome numbers still grow, must be of advanced age. They must certainly be much older than polyploid species with chromosome numbers which are topping a complex, but which still grow in the near neighbourhood of, or together with, their ancestors. This particularly applies to polyploids growing in areas which have been subject to heavy climatic oscillations, such as Europe during Quaternary and post-glacial times.

Returning to the Arctic, we shall study a polyploid complex represented here, with a long series of high chromosome numbers, such as the *Papaver radicatum* complex. It has a series of numbers ranging from 14 to 84, with basic number 7. It is striking that only the highest chromosome numbers 56, 70 and 84 belong to the European sector of the Arctic, whereas also the lower numbers are found in America, preferably in the western part, and in Asia. There is a marked differentiation as to phenotype and chromosome structure at each polyploid chromosome level, which shows that the polyploids must be of great age in the European area (KNABEN, 1959). We must suppose that they migrated there in pre-Quaternary or inter-glacial times, and that the populations became split during the glaciations.

This part of the Arctic owns other genera or subgenera with isolated polyploids, which are now far removed from their low-numbered relations, or without relations in other areas: *Arenaria ciliata* subsp. *pseudofrigida*, *A. humifusa* and *A. norvegica*, *Braya linearis* and *B. purpurascens*, *Ledum palustre*, *Primula scandinavica* and *P. stricta*, *Nigritella nigra*, *Platanthera oligantha*, *Saxifraga hieraciifolia*, etc. Similar to the high polyploid Papavers, these polyploids may have migrated to this part of the Arctic in pre-glacial or inter-glacial times.

The old arctic-alpine elements of the Scandinavian flora, which most possibly are glacial survivors, have, like the old diploid species in the Alps, spread very little in post-glacial times. They seem to grow nowadays not very far from where they hibernated, either in ice-free coastal strips or on nunataks farther inland. It also seems as though, like the diploid species in the Alps, they must have wandered in groups, seeing that today they are found isolated on the same mountains. Strikingly, all, or most of them, are little variable. This is the case with regard to both the diploid and polyploid members. All these conditions suggest that both categories of plants, diploids and polyploids, like the diploids in the Alps, are of great age within the Scandinavian area, indicating

that polyploid species of an old flora element have not the same faculty of colonizing and spreading as a newly arisen, "raw" polyploid type.

2. The lowland flora groups

Considerable importance has been attached to the great faculty of newly arisen polyploids to colonize. Analyses show that they must have far greater ability to occupy new areas than their diploid relations. Their new, unstabilized gene combinations have much selection value on virginal soils. They evidently have less ability to penetrate into a ripe vegetation cover.

In late glacial and post-glacial times there were in Europe several categories of new soils ready for colonization. Firstly, there were the areas laid bare after the melting of the ice, both in the lowlands and in the mountains. Later, there were the shores along the coasts and along the large inland lakes, which rose above sea level, subsequent to the general elevation of the countries in post-glacial times.

It is supposed that at the beginning of the Quaternary period Europe, north of the Alps, had a flora of about the same kind as today. This flora hibernated on ice-free refuges to the north-west, south and east of the ice cover, and migrated back again, when the climate improved, shortly after the retreat of the ice.

We shall consider briefly how the different climatically conditioned flora groups of Europe vary in distribution according to the variation in climate today. The variation today, in the climate in Europe changing gradually northwards, concerns firstly the light conditions. The change in day and night lengths during the year differs very much from south to north. The temperatures also vary with the latitude, but the climate in Europe, both with regard to temperature and moisture, depends more upon the situation of the areas in relation to the large mountain ranges and their direction, and then, on the distance of the areas from the Atlantic Ocean. In Western and Central Europe the conditions change along a west—east gradient.

On TROLL's map (Fig. 2) three zones are marked in Western Europe which are influenced by the Atlantic Ocean with decreasing effect eastwards: the eu-Atlantic zone along the Atlantic coast, the sub-Atlantic farther inland, and the eury-Atlantic zone penetrating in between the sub-arctic coniferous tree region to the north-east and the arid region with steppe vegetation to the south-east. The oceanic flora elements of these three zones are in Norway concentrated in the coastal region of

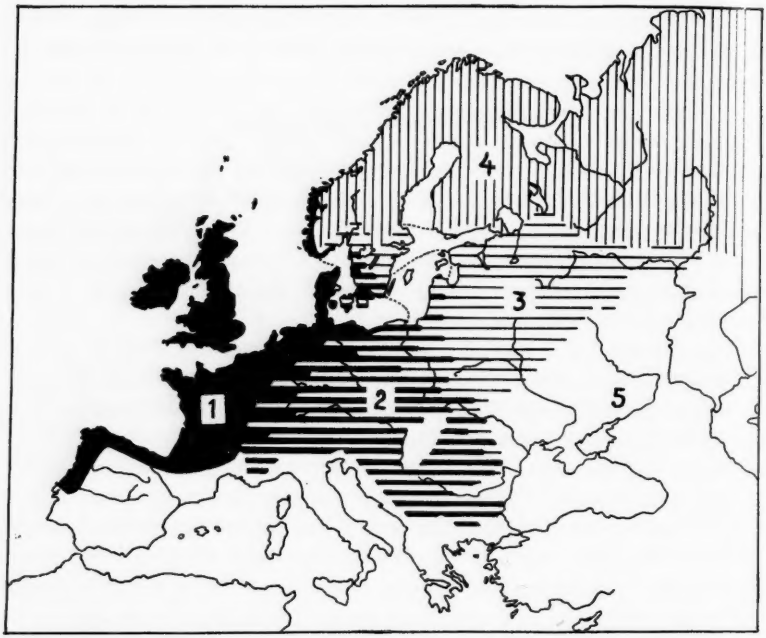

Fig. 2. Reprint of Troll's map of floristic regions in Europe. 1: Eu-Atlantic, 2: Sub-Atlantic, 3: Eury-Atlantic, 4: Boreal and Arctic, 5: Semi-arid regions.

Western Norway. They are characterized by a northern limit going farther to the north in Western Norway than in the warmer and drier districts in Eastern Norway. The latter districts for their part have — as character plants — an element of thermophilous and xerophilous species growing preferably on edaphically good localities. Many of them show connections with the arid steppe vegetation of south-eastern Europe.

We shall not consider in detail the distribution of the climatically conditioned plant groups elsewhere in Europe, but only mention that the sub-oceanic plants in the Mediterranean climb up on the mountain sides; these therefore have been noted as sub-oceanic montane species.

It seems obvious when investigating occurrence and significance of polyploidy in Europe to base this on our knowledge of the distribution of the climatically conditioned plant groups, the recent wanderings of which have been discussed for a century, i.e. since AXEL BLYTT (1876)

formulated his theories of the immigration of the Norwegian plant groups in alternating dry and rainy periods in post-glacial times.

I have computed the percentages of polyploid species by different groupings of our lowland species.[1] We see that the group of lowland species has a very low proportion of polyploid species, either their northern limit falls in Nordland at about the Arctic Circle, in Trøndelag, or we consider only the species in South-Eastern Norway, south of the Dovre range (Groups 5—9, Tables 3 and 4). The two latter groups of species (8 and 9) correspond to BLYTT's boreal and sub-boreal plants.

The dominance of diploid species in the southern and south-eastern flora elements disagrees with the theory that it is the autochtone floras which include most diploids. Analysing the contents of species it appears that these groups comprise many genera with little or no faculty of polyploidy. On the whole, 35 of the south-eastern species belong to different genera with solely diploid numbers. Of these, 14 genera are monotypic and not variable. It is remarkable that all the diploid species of the south-eastern group belong to genera with the northern limit in Southern Norway, and that this is the case with many of the polyploid members of the groups, too. It is not only the species, but the genera to which they belong that have their northernmost findings in Europe in Southern Norway. The south-eastern species represent a characteristic, alien element in our flora, behaving as if they were relics from a warmer period. Now they occur in the warmest and driest parts of the country. The many diploids must have roots back in the European Tertiary flora. They adjusted themselves during the changing conditions in the late Tertiary and Quaternary periods at the outskirts of the ice sheet, and after the retreat of the ice, in a favourable climatic period, wandered northwards again, probably together with other steppe plants. Here they found environments of about the same type as those to which they had become accustomed, and that is where they grow today.

It was mentioned above that recent investigations of the glacial flora in Central Europe have revealed that the Quaternary climatic oscillations were followed by a vivid formation of polyploids within many genera after the melting of the ice. An obvious question here is whether the Norwegian flora which came from the south is characterized by polyploid types of Quaternary or perhaps post-glacial origin. The

[1] The grouping of the species is based on the maps of distribution in HULTÉN's Flora Atlas and on the lists of species worked out for the mapping of the Norwegian flora by FÆGRI, GJÆREVOLL, LID and NORDHAGEN.

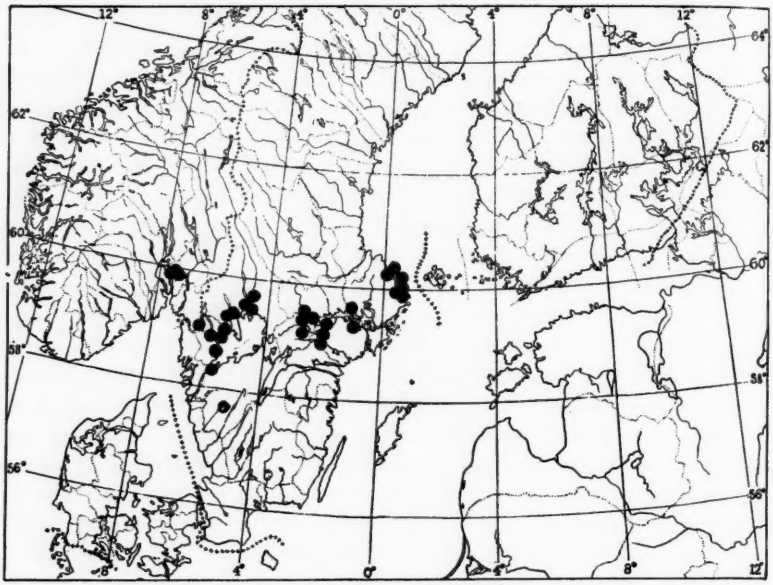

Fig. 3. The distribution of *Saxifraga osloënsis*. From KNABEN (1954).

answer must be that we certainly have such types. Some of them have arisen farther south in Europe, and reached us in company with ecologically similar, older species, whereas others have arisen when the boreal — sub-boreal and arctic — sub-arctic flora groups met each other in Southern Scandinavia during migratory times.

We will study some examples. Most *Galium* species show intra-specific polyploidy in Europe. Two species belonging to our southern lowland group, *Galium mollugo* and *G. verum*, came from South-Eastern Europe. FAGERLIND (1937) dealt with these two species. In Norway they grow in somewhat changing environments on dry slopes and on crags. In South-Eastern Europe both show intra-specific polyploidy.

FAGERLIND investigated the geographical distribution of the chromosome numbers. He found that the diploid types grow only in South-Eastern Europe, in some places together with the tetraploids, whereas he only found tetraploids to the north of the Balkans and the Carpathians. Populations from about 70 localities were investigated, from the Balkans and Transcaucasia, northwards to southernmost England and the Stockholm area.

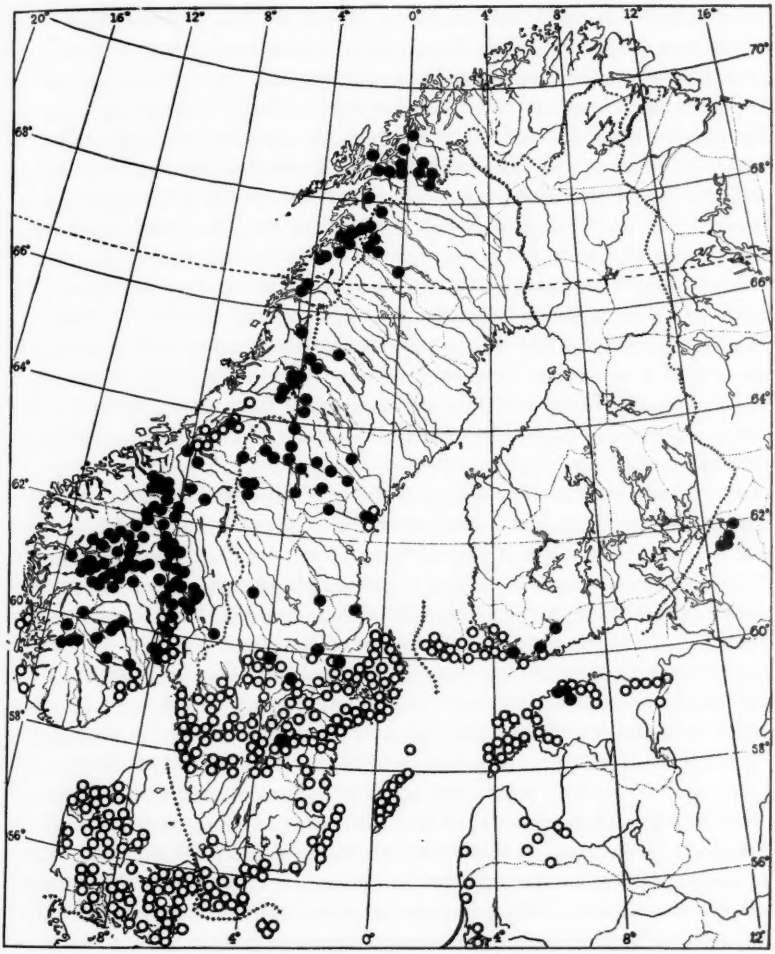

Fig. 4. The distribution of *Saxifraga adscendens* (●) and *S. tridactylites* (○) in Fenno-Scandia. From KNABEN (1954).

It must be supposed that probably the polyploid types arose during or after the Quaternary period, either from inter-variatal hybrids, or through a purely quantitative increase in chromosome numbers. Judged from the distribution of the chromosome numbers it seems that the diploids have not been able to spread northwards, whereas the tetra-

ploids have colonized the land laid bare after the melting of the ice. In Norway we will certainly find only tetraploids of both species, which, in accordance with modern theories, we can consider as relatively young categories of plants. The two *Galium* species have a little wider distribution in Southern Norway than the species of BLYTT's boreal and sub-boreal groups. In South-Eastern Europe, diploid and tetraploid *mollugo* are ecologically very alike, growing among each other in the localities, whereas diploid and tetraploid *verum* are found in ecologically different niches. The two species must have reached Norway in company with the south-eastern, temperate flora element, which migrated into Scandinavia shortly after the retreat of the ice when the climate suddenly improved. Sub-fossil deposits in southernmost Sweden show that a sub-arctic flora with pines overtook the arctic *Dryas* flora of late glacial times, and that the temperate flora from the south-east followed the sub-arctic flora before the spruce invaded the country on a broad front.

In Scandinavia we have a polyploid species in an area where the south-eastern flora must have met the sub-arctic, and perhaps also some arctic plants which had followed the retreating ice. This species, *Saxifraga osloënsis*, is regarded as an amphidiploid, arisen after chromosome doubling in the hybrid *S. adscendens*×*tridactylites* (KNABEN, 1954). *S. osloënsis* (Fig. 3) grows across Southern Sweden, preferably near the great lakes. In Norway it is found only in a restricted area at the head of the Oslofjord, and almost only on land laid open for colonization after the elevation of the country at the end of the post-glacial warm period (BLYTT's Sub-Boreal period). It grows below the marine limit in Oslo and its surroundings. The supposed ancestors: sub-arctic *adscendens* and temperate *tridactylites* (Fig. 4) still grow within the area of *S. osloënsis*. *S. adscendens* is rare in this lowland area, where it occurs at its southern limit in Scandinavia, whereas *tridactylites* grows abundantly and in company with *osloënsis* in the localities. *S. tridactylites* belongs to our boreal group of plants with connections to the south-east in Europe.

3. The oceanic species

Before considering other possible sources of our eastern polyploid lowland species, we shall consider briefly the occurrence of polyploids in the coastal flora. FÆGRI's group of coastal plants in Table 4 is not very appropriate for our purpose. It comprises the species with coastal

occurrences which FÆGRI (1960) treats in the first part of Norway's Flora Atlas, just published. All Norwegian coastal plants are included in this group even if the species have a rather eastern distribution farther south in Europe. The present author has separated two narrower groups from this larger group. One includes coastal plants along our western coast with oceanic distribution also farther south in Europe, the other includes the species which have a similar distribution in Norway to that of *Bromus Benekenii* (cf. below p. 472).

There are 42.5 % polyploids in the group of pronounced oceanic species of Western Norway. It is a high percentage, the mode of calculation taken into account. In accordance with the points of view maintained above, the question is whether this high percentage can be explained in accordance with modern theories. The moist coasts along the Atlantic Ocean, with cool summers and mild winters and heavy rainfall, formed new land with new kinds of localities, which became ready for colonization during the post-glacial rainy periods. This new land differs in our time very much from the soils and climatic conditions to which the late Tertiary flora had adapted itself during the Quaternary period.

We shall notice that in the oceanic element there are as many as 12 of the species belonging to diploid or almost purely diploid genera, lacking the faculty of polyploidy, which have spread to the moist coasts, adapting themselves without any change in chromosome numbers. It is further noticeable that all the oceanic polyploids belong to variable genera rich in differing taxa, with series of related chromosome numbers, all of which occur today within the oceanic-influenced areas of Scandinavia or farther south in Western Europe.

These conditions are suggestive of the occurrence within the oceanic zones of relatively young polyploid species that arose in post-glacial times. If we wish to study a polyploid complex in the Atlantic zones of Europe, the *Luzula campestris-multiflora* complex is a good example. It comprises the following series of chromosome numbers: 12, 24, 36, and 48 distributed in Western Europe today.

The species, *L. congesta*, with 2n=48, which is the highest polyploid of the complex, is eu-Atlantic, restricted to the outermost coastal strip from Western Norway to Spain. It belongs to the strongly humified zone developed in post-glacial times. It may be a polyploid of recent origin. However, as shown by HEDDA NORDENSKIÖLD, all the *Luzulas* are in reality so variable in Western Europe today, with series of related types varying as to chromosome numbers, that we ought to discuss

whether "a recent wave of polyploidy" has not taken place within the genus, to use IRENE MANTON's mode of expression.

A pair of species, *Cardamine hirsuta* and *C. flexuosa*, may be mentioned in this connection. The first is diploid, the latter tetraploid. Both species are oceanic-accentuated as to distribution, but only the tetraploid is regarded as distinct oceanic. The diploid, *hirsuta*, is included among the species with distribution in Norway similar to *Bromus Benekenii* (FÆGRI, 1960). The *Bromus Benekenii* group has a lower percentage of polyploids than the more pronounced oceanic species: 16.2 % as against 42.5 % in the latter. The *Bromus Benekenii* group of species does not, like the distinct oceanic, reach the outermost coastal strip, but is confined edaphically to better localities farther inland. It is strange that 15 of the 18 species of the *Benekenii* group are diploids. The group includes 20 species, but two are not known as to chromosome numbers. From a cyto-ecologic point of view we could perhaps also include one of the three polyploids among the group of diploids, viz. *Hedera helix*. Certainly, it is a tetraploid species in the genus; in Australia there is a species *H. australiana* with 2n=24 chromosomes as against 2n=48 in *H. helix*. But it has the lowest chromosome number of the European species of *Hedera*, and it has the widest distribution across the European continent. On its borders to the west and south there are octoploids, *H. hibernica* in Ireland and *H. canariensis* in the Mediterranean and the Canaries, both with 2n=96. To the east a species with 2n=192 is known, viz. *H. colchica* in Caucasia and Persia (JACOBSEN, 1954). Accordingly, provided that *H. helix* has 2n=48 throughout Europe it may be a tetraploid of advanced age in Europe, and probably the progenitor of relatively younger polyploids on the borders of its area.

The question is whether the restricted distribution of the *Benekenii* group in Western Norway is connected with old, diploid or higher, chromosome numbers. The species are no longer sufficiently variable and cannot adjust themselves to new conditions. It is often seen that species on the border of their area are restricted to the edaphically better localities. The *Benekenii* species were probably crowded out and pressed back to their localities of today when the soils in the outermost coastal region were heavily leached out in the cool climate with high rainfall which has favoured the formation of acid raw humus.

If it be correct that the distribution of the *Benekenii* species can today be explained by supposing that they were not able to adjust themselves to the new conditions, the distribution of the two species *Cardamine*

hirsuta and *flexuosa* can be understood. *C. hirsuta*, itself a diploid, can then be said to have a tetraploid descendant, *C. flexuosa*, as a substitute in the pronounced oceanic element. It is evident that the tetraploid in this case has been able to penetrate into new and strange localities, whereas the diploid has had more restricted possibilities. In Southern Europe *C. flexuosa* is oceanic-montane, whereas *hirsuta* is more restricted.

V. CONCLUSIONS

Summing up the results hitherto attained of the analyses of the Norwegian flora groups, it seems clear that in order to provide a logical explanation of the variable distribution of the polyploid species, the floras have to be analysed separately as to content of polyploid complexes in relation to various factors. Especially the Quaternary history of the plants, the stations where they hibernated, their migratory ways and the long distances they may have travelled, in addition to their adaptability to climatic oscillations have been factors of great importance.

We must be aware of the fact that our oceanic flora element may include recently arisen polyploids, just as our eastern flora or the whole lowland flora, probably comprises such polyploids. This being so, it seems that many phyto-geographical problems concerning the Norwegian flora, which have been discussed for a long time but have hitherto been difficult to elucidate, can now be given a logical explanation.

Finally, in support of these points of view, some of the results of the above-mentioned investigations during the last decade of the chromosome conditions of the glacial flora of Central Europe in relation to distribution will be briefly cited, especially those of interest for our lowland flora.

Surprisingly enough, it would appear that some of our lowland species originate from the ancient flora of the Alps, viz., from the local age-old element of diploid species. These diploid species have, through intra- or inter-specific polyploidy, been the source of new types, which wandered down into the lowland and occupied the areas north of the Alps. As FAVARGER has stressed, for alpine species the life of lower heights, on the plains, represented new conditions. It seems now to be proved that many polyploid races of a series of alpine species have spread down into the lowland, unlike the diploids themselves, which still grow where they hibernated in the peripheric Alps.

It has long been known that the Scandinavian arctic-alpine flora is related to the flora in northern arctic areas, and that it has less connection with the flora of the Alps. Against this background it is surprising to see that the lowland flora has been recruited from the flora in the Alps. SÖLLNER and FAVARGER, in 1950 and 1954, were the first to assert that diploid alpine species have given birth to polyploid lowland races. It was races of *Cerastium arvense*, *Lotus* species and of the *Anthoxanthum odoratum-alpinum* complex that they discussed.

As to *Cerastium arvense*, we no longer consider, as HEGI did, that its subsp. *strictum*, with restricted distribution, is an alpine ecotype, separated from subsp. *commune* that grows abundantly in the lowland north of the Alps. On the contrary, we maintain that the polyploid, widely dispersed subsp. *commune* originates from the alpine type. In 1959 FAVARGER showed that, within *Aster alpinus* and *Chrysanthemum leucanthemum* polyploid races had similarly arisen through intraspecific polyploidy, and that the diploid ancestors were found in the relic alpine flora in the refuge area of the Alps. The latter, of interest to our flora, has diploid, tetraploid, hexaploid and octoploid races in the European glacial area. However, it must be noted that besides the alpine diploid race studied by DUCKERT and FAVARGER, occurring today on the western brim of the Alps, there is also a diploid growing at the present time on the plains in Central Europe northwards to Denmark and Southern Sweden. This diploid race may have hibernated in the refuge area in the north-western part of the Continent or in West Jutland (BØCHER and LARSEN, 1957).

BØCHER and LARSEN show that the specific name *Leucanthemum* must probably be retained for the diploid race, as the specimen belonging to the Linnean Herbarium of the Linnean Society in London accords as to phenotype with a diploid specimen. The *Leucanthemum* complex has not yet been unravelled. In Norway we probably have one or more of the polyploid races.

EHRENDORFER's investigations of the *Galium pumilum* complex are of interest for the present analyses. In this complex it is also the diploid which has restricted, and in this case, disjunct, distribution in the peripheric alpine nunatak-area, whereas the tetraploid, hexaploid and octoploid races have a much wider distribution in the lower parts of Central Europe. The distribution of the polyploids solely in the once glaciated areas of Europe suggests also in this case that the race formation has taken place during or after the Quaternary climatic oscillations.

In Norway there are two races of *Galium pumilum*, one in the north,

on the island of Vega in Nordland (subsp. *Normanii* O. DAHL), the second in a small area in South-Eastern Norway. Their chromosome numbers are unknown, but probably they are polyploid. The same reasoning can be applied to *Galium palustre* and *G. uliginosum*, which both show intra-specific polyploidy. In England, HANCOCK (1942) has found both diploid and polyploid races.

The lowland localities in Norway must be regarded as terminals for the wanderings of the species occurring here after the Ice Age. It would be of great interest to ascertain whether the genera contain relatively more polyploids and less diploids in our area than farther south and east, as a result of a connection between increase in polyploidy and long distances traversed, and between polyploidy and the faculty of colonizing. For the present we can only conclude by stating that in the lowlands, both within the eastern xerophile flora and within the oceanic group, there exists, besides an ancient diploid element, a polyploid element which may contain species of recent origin.

Similarly, the arctic-alpine flora contains a multitude of still unsolved problems. The *Empetrum nigrum* problem must be reconsidered owing to FAVARGER and his collaborators having found the tetraploid *hermaphroditum* in the Alps. Previously, it was only known in northern areas. The diploid *E. nigrum* grows all over the lower parts of Europe, where *hermaphroditum* is missing or rare. The latter is an arctic-alpine species, probably belonging to an ancient flora element. Nor is the *Antoxanthum* problem easily explained. In contrast to the Empetra, the diploid *Anthoxanthum* races are arctic-alpine, with the tetraploids in the European lowland. The history of these two polyploid complexes is, perhaps, not easily unravelled.

In addition, we have all the problems concerning common species, with varying ecology, or with no clear connections with other areas. Some of them have been subjected to the searchlight of our phytogeographers, such as the mountain birch met with in a belt above the coniferous forests of spruce and pine. On Scandinavian mountains and in the European Arctic it is the mountain birch that dominates the forests in a belt at the upper forest limit. The mountain birch forest has been characterized as an Atlantic phenomenon from post-glacial wet periods. It constitutes a vegetation type without relations far back in time or with other areas, the origin of which has been difficult to explain.

The mountain birch is not only ecologically distinctive. It is morphologically fairly well separated from the birch at lower heights. Scandi-

navian botanists have considered it as specifically distinct. It has been named *Betula tortuosa* LEDEB., to distinguish it from *B. pubescens* coll. at lower heights. Both *B. tortuosa* and *B. pubescens* are polyploid, with the same number of 2n=56 chromosomes. *B. verrucosa* EHRH. in regio silvatica and *B. nana* L. in regio alpina are both diploid, with 2n=28.

In our country, up to the present time, problems of plant distribution of this nature have been approached according to the descriptive methods. Even if this older method is far from being exhausted, the time is perhaps now ripe for attacking the problems according to modern methods, basing the work on cyto-genetic characters. Hitherto, it has been first and foremost the aristocrats in our flora, the rare mountain species and the ecologically clearly limited groups of species, which have been analysed as to their migratory history and origin, and also from a chorological point of view. An intensive study with chromosome counts of the more common, trivial plants will now, it seems, certainly assist in throwing light upon problems concerned with the origin of our flora as a whole.

The present account cannot be terminated without citing STERN's (1946) work on *Paeonia*, a widespread genus in the northern hemisphere. STERN's conclusions with regard to the distribution and significance of polyploidy in this genus accords with the present author's views. STERN thinks that the distribution of the species today must be due to changes which took place in the Ice Age. In Europe, the oldest species, that is the diploid species, are only found in Portugal, in the islands of the Mediterranean, in the Crimea and Volga regions, and in a small area in the Caucasus. The distribution of these species suggests that they are pre-glacial relics from which tetraploid species arose. These latter, new species, have proved themselves better adaptable to post-glacial conditions than the old diploid species. They have spread to areas which were covered by the ice sheet and laid bare after the melting of the ice. Here their diploid near relatives do not grow. An example is *P. daurica*, which occurs in the Crimea and the surrounding neighbourhood, whereas allied tetraploid species, *P. mascula* and *P. banatica*, stretch westwards across Europe, through Hungary and Germany to France.

Manchuria and China were not covered by the ice sheet. In these countries nine paeonie species are found; they are all diploid and are considered to be pre-glacial relics. Likewise, in California in North America, diploid paeonie species are found in areas which were not glaciated. They also are considered as ancient relics which have de-

scended from the same source as the species in Western China. It is curious, STERN says, that in Asia and America all the species are diploid, with only one known tetraploid, whereas in Europe there are more than twice as many tetraploid species as diploid species.

In the genus *Pyrola* there is only one known tetraploid species, viz. *P. media*. It is supposed to have arisen as an amphidiploid from the cross between the two diploid species *P. minor* and *P. rotundifolia* (HAGERUP, 1941; KNABEN, 1943). It grows today, similar to the tetraploid paeonie species, in once glaciated areas, viz., in Europe, North Asia and Asia Minor, but not in Central Asia, Manchuria and Japan. The family *Pyrolaceae* is thought to have had its origin in the latter areas, which appear to be rich in *Pyrola* species. *P. minor* and *P. rotundifolia* must have emanated from there. The distribution of *P. media* has hitherto been difficult to explain. If the species is considered to be of glacial or post-glacial origin, arisen during periods of upheavals caused by changes during the Ice Age, then its distribution can the more easily be understood.

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CHROMOSOME STUDIES ON FINNISH PTERIDOPHYTA II

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INTRODUCTION

INTEREST in the cytology and cytotaxonomy of the Pteridophyta has greatly increased during the last ten years over the entire world. This can be clearly seen from the indexes compiled by ALAVA, ARNOTT, BELL *et al.* (1956—1959), and from the large list of Pteridophyta chromosome numbers by CHIARUGI (1960). Clear cases of polyploidy were found in many families and genera. Even in the same species several cytotypes were found, for example, in *Ophioglossum vulgatum* and *O. reticulatum* (ABRAHAM and NINAN, 1954; NINAN, 1958 b; VERMA, 1956, 1958).

The highest chromosome numbers in the Pteridophyta were mostly found in the tropical and subtropical zones. The highest degree of polyploidy usually seems to be reached in the middle of the distribution area. In the border regions, on the other hand, there are only lower-numbered cytotypes. The last Ice Age obviously disturbed the normal distribution of the Pteridophyta species in north-western Europe, and perhaps some new polyploid species originated in that glacial period, as supposed by MANTON (1950). The different frequency of polyploidy between the leptosporangiate ferns of the British Isles and Madeira may also be caused by the fact that many of the Madeira species are tropical, and as such lie on the border of their geographical area. Similarly, many fern species, which have higher numbered cytotypes in Central Europe and the British Isles, are only diploid in Scandinavia and Finland.

The meiosis of seven species of the Finnish Pteridophyta was studied cytologically. The specimens were collected in South Finland, near Helsinki, during the summers of 1957—1960. As they were collected the young sporangia were immediately fixed by using Carnoy's (1:3) acetic alcohol. The time of fixation varied from 1 to 24 hours. After long fixation the sporangia were kept in 45 % acetic acid about an hour before

staining with acetocarmin (Riedel-de-Haen). The time of staining varied from 1 to 2 days. Squash preparations were pressed with a strong "foot-press" and then moved through the alcohol series to Euparal mounting medium. Microscopying and camera lucida drawings were carried out with Wild KGM 20 microscope (objective Fluotar 100×, oil-immersion, A=1.30, and 15× kompens oculars). The phase contrast optics of Wild were also used for inspecting difficult objects and for counting the chromosome numbers.

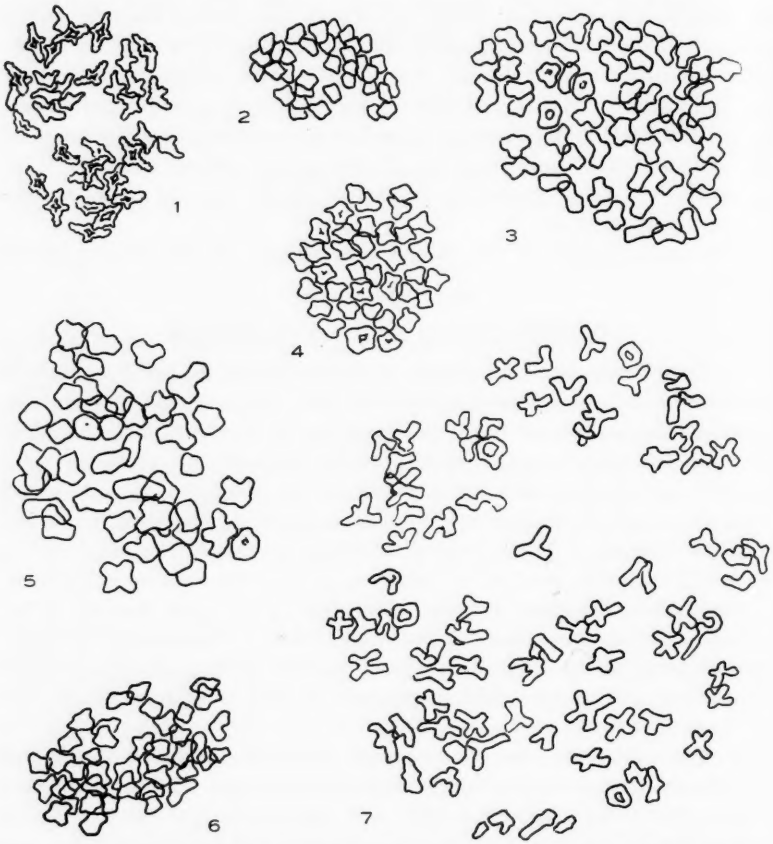
The nomenclature of the species corresponds to that of HYLANDER (1955).

OBSERVATIONS AND CONCLUSIONS

Lycopodiaceae: Two species of Finnish club mosses *Lycopodium annotinum* L. and *Lycopodium clavatum* L. (Figs. 1 and 2) were studied. In the meiosis of both these species 34 bivalents were counted. The same number was earlier determined by MANTON (1950) in material from the British Isles, Switzerland and Sweden. MEHRA and VERMA (1957 a) counted 34 bivalents in diakinesis of *L. clavatum* from the Eastern Himalayas, Darjeeling district; LÖVE and LÖVE (1958) determined the somatic number 2n=68 from the Canadian plants of *L. annotinum* and *L. clavatum*. The same number n=34 is also known in the species *L. obscurum* and *L. nikoense*. All these species seem to be tetraploids, x=17 being the probable basic number of the genus, but higher numbered species (n=136) are known in this family in the tropics (NINAN, 1958 a).

Polypodiaceae: In the Common Bracken *Pteridium aquilinum* (L.) KUHN (Fig. 3) there are 52 bivalents in meiosis. The same number was earlier determined by many other cytologists, for example, MANTON (1950) in material collected from Malaya and the British Isles, and by MANTON and SLEDGE (1954) in the plants from Ceylon. BRITTON (1953) and WAGNER (1955) counted the same number in two North American variations of *Pteridium aquilinum*; BROWNLIE (1957) counted it in var. *esculentum* from New Zealand; and lastly MEHRA and VERMA (1960) found it in var. *wightianum* from the Western Himalayas. All observations up to the present time would indicate that the chromosome number of this cosmopolitan species is very uniform compared with most other fern species.

A Spleenwort species *Asplenium trichomanes* L. (Fig. 4) was investigated. All specimens hitherto studied have proved to be diploid. There are 36 bivalents in meiosis. In Central Europe this fern species has



Figs. 1—7. The SMCs of the Pteridophyta during diakinesis and the first metaphase. — Fig. 1, *Lycopodium annotinum* L. n=34. Fig. 2, *Lycopodium clavatum* L. n=34. Fig. 3, *Pteridium aquilinum* (L.) KUHN n=52. Fig. 4, *Asplenium trichomanes* L. n=36. Fig. 5, *Athyrium filix-femina* (L.) ROTH. n=40. Fig. 6, *Woodsia ilvensis* (L.) R BR. n=41. Fig. 7, *Cystopteris fragilis* (L.) BERNH. n=84. Acetic alcohol+aceto-carmin squashes, magnification about ×1500.

both diploid and tetraploid sporophytes. The tetraploid form, which may be more common compared to the diploid, has been found by many cytologists in Europe, for example, MANTON (1950), MEYER (1952, 1957, 1958 a), FABBRI (after CHIARUGI, 1960); in North America, BRITTON (1953); and in the Western Himalayas, MEHRA and BIR (1957). The

first observation of a diploid *A. trichomanes* was published by MANTON in 1950 from Wales. Thereafter the diploid cytotypes were found in Europe by several authors (MEYER, 1952, 1957, 1958 a, b; LOVIS, 1955; VAZART, 1956); and by BRITTON (1953) from North America.

Some of the diploid specimens from Finland mentioned above were fixed from the same rock in Stansvik near Helsinki as the tetraploid *Asplenium septentrionale*, studied and published by the present author some years ago (SORSA, 1958). The hybrid of both these species (*A. germanicum*) was also formerly found at the same site, but it was not studied cytologically in Finland. MANTON (1950) determined the chromosome number of *A. germanicum* (2n=c. 108) from Welsh, Italian, Swedish and Swiss material, and later the same triploid number of chromosomes was counted again by MEYER (1952, 1957, 1958 a, b) from Central Europe. MEYER (1957, 1958) also found a hybrid of *Asplenium septentrionale* and tetraploid *A. trichomanes* (2n=144) from Germany. Hybridization between the diploid and tetraploid forms of *A. trichomanes* seems to be also possible. LOVIS (1955) found such a hybrid *A. trichomanes* (2n=108) from the British Isles, and BROWNLIE (1954) recorded obviously the same hexaploid hybrid from New Zealand. The meiosis in the hybrids seems to be irregular.

To indicate the diploid and tetraploid cytotype of *A. trichomanes* the cytological determination of the chromosome number is of course most reliable. However, good results were obtained by using only morphological identification, for example, the spore measurement, which was proved to be suitable even for determinations from herbarium material (LOVIS, 1955).

The Common Lady Fern, *Athyrium filix-femina* (L.) ROTH (Fig. 5) is diploid in Finland. There are 40 bivalents in meiosis. The chromosome number of this fern species had been counted by FARMER and DIGBY (1907), who calculated n to be 38—40, but the exact number was first determined by MANTON (1950). The same number (n=40) was then found by BRITTON (1953) and WAGNER (1955) in a North American variation (*michauxii*) of *A. filix-femina*. In India many other species of *Athyrium* were investigated by MEHRA and VERMA (1957 b). Most of them seem to be diploids with only two tetraploid species counted. In Ceylon, on the other hand, also hexaploid and octoploid species were found by MANTON (1953, 1954), and MANTON and SLEDGE (1954). Another basic number (n=41) was also found in some *Athyrium* species from Ceylon (MANTON, 1954) and from New Zealand (BROWNLIE, 1958).

Woodsia ilvensis (L.) R. Br. (Fig. 6) seems to have about 41 chromosome bivalents in the SMCs during first meiotic metaphase. The same number was earlier counted by Manton (1950) in material from Wales, and Britton (1953) in material from Southern Ontario, Canada.

The chromosome number of the cosmopolitan fern species *Cystopteris fragilis* (L.) Bernh. (Fig. 7) was counted for the first time at the end of last century by Stevens (1898), and about forty years later by Lawton (1936). However, the exact number (n=84) was first determined by Manton (1950) in material from the British Isles. Manton also found a higher numbered (n=126) cytotype of this species. The same hexaploid number was later counted by Wagner (1955), and Wagner and Hagenah (1956 b) in a North American variation (*tennesseensis*) of *Cystopteris fragilis*. In Finland this fern species seems to be tetraploid, having 84 bivalents at the first meiotic metaphase. The tetraploid number was earlier determined in plants from Ontario, Canada (Britton, 1953) and from Michigan, USA (Wagner, 1955). The only diploid variation (*C. fragilis* var. *portuosa*) was found in the same region from southern Michigan (Wagner, 1955; Wagner and Hagenah, 1956 a).

Most of the species of Pteridophyta found in Finland have a wide distribution in the southern as well as in the northern hemispheres. The determinations of the chromosome numbers indicate that many species have several cytotypes, especially in the middle of their geographical area. In the border regions like Scandinavia and Finland usually only one, lower numbered cytotype can be found in most species. This can be best seen in the following table.

Four of the fourteen Finnish fern species mentioned in the table have higher numbered cytotypes in the southern parts of Europe. It is not known why these polyploid forms have not spread to the northern regions. Maybe they originated in the last Ice Age, and have not yet had sufficient time. Perhaps the ancient, lower numbered cytotypes are better adapted to the extreme conditions of the North.

It seems that the correlation between the relative percentage of polyploids in the flora and the geographical latitude is not as clear in the Pteridophyta as among the Spermatophyta (Löve and Löve, 1948). The Pteridophyta of the tropical regions seem to have more higher numbered cytotypes than those of the temperate climates. This problem was considered by Manton (1955), who suggested that the percentage of polyploids is higher in the Pteridophyta flora of Ceylon compared to that of Madeira or the British Isles.

The different known cytotypes of the fern species studied from Finland.

Species	Cytotypes found in Finland	Cytotypes found elsewhere in Europe
Polypodium vulgare	4n	2n, 4n, 6n
Eupteris aquilina=Pteridium aquilinum	2n	2n
Asplenium trichomanes	2n	2n, 3n, 4n
A. septentrionale	4n	4n
Athyrium filix-femina	2n	2n
Dryopteris spinulosa	4n	4n
D. austriaca=D. dilatata	2n	2n, 4n
D. filix-mas	4n	4n
D. thelypteris=Lastrea thelypteris	2n	2n
D. linnaeana=L. dryopteris	4n	4n
D. phegopteris=L. phegopteris	3n	3n
Woodsia ilvensis	2n	2n
Cystopteris fragilis	4n	4n, 6n
Struthiopteris filicastrum=Matteuccia struthiopteris	2n	2n

The most probable explanation of this quite different distribution of the polyploid forms of Pteridophyta and Spermatophyta is obviously the different kind of sexual reproduction in these plants together with the different mode of interchange of generations. In the tropics and subtropics the sexual reproduction of Pteridophyta must occur more successfully and more frequently than in the temperate zones. For that reason also, the frequency of hybridization must be higher in the tropics, and new alloploid or autoploid forms may arise more easily than in the cold climates. Under these favourable conditions and environments, much resembling those of the ancient flourishing periods of the group, the evolution of Pteridophyta can progress faster in the tropics than in the more difficult conditions of the temperate and cold climates.

The sexual reproduction and hybridization of the Spermatophyta are less dependent on climatic conditions. Thus new polyploid forms can arise more frequently among the highly adapted species in the border regions of their distribution area. Maybe it can occur even more readily than in the thick rain forests. Because of the greater reserve of allelic genes the polyploid cytotypes usually succeed better in difficult conditions. They are able to spread and adapt themselves to the outermost regions. Thus their relative percentage is directly correlated with the distance from the equator (TISCHLER, 1935; LÖVE and LÖVE, 1943, 1948).

Another interesting problem in the cytology of the Pteridophyta is

the very regular formation of bivalents in meiosis of the highly polyploid species, for example, *Ophioglossum reticulatum* (ABRAHAM and NINAN, 1954; NINAN, 1958 b). To explain this, the hypothesis has been put forward that polyploid forms are alloploids of ancient species. The abundance of cross-shaped bivalents in diakinesis of the higher numbered polyploids points to a low frequency of chiasmata or a strong chiasma interference in those species. Maybe there is a mechanism, for example, the localization of chiasmata, that prevents the formation of multivalents in meiosis. On the other hand, in some European hybrids of the fern species univalents and multivalents can be seen.

SUMMARY

Seven species of Finnish Pteridophyta, two lycopods and five ferns, were studied cytologically. The chromosome numbers were found to be the same as earlier recorded in these species in the other parts of Europe and elsewhere. *Asplenium trichomanes* seems to be diploid in the present material collected from Finland. The tetraploid *A. septentrionale* and the hybrid species *A. germanicum* have been found in the same region as the diploid *A. trichomanes*. Some of the species of Pteridophyta seem to have higher numbered cytotypes in Central Europe than in Finland.

The correlation between the percentage of the polyploids and the geographical latitude is not as clear as found in the Spermatophyta. On the contrary, many Pteridophyta species have lower numbered cytotypes in the border regions and higher numbered cytotypes in the middle of their distribution. In the tropics and subtropics large series of polyploidy were found in many species of ferns. In my opinion this difference in the distribution of the Pteridophyta and the Spermatophyta can be caused by the different interchange of generations and sexual reproduction in these plant groups. Cold and temperate climates suppress the hybridization and evolution of the Spermatophyta to a lesser degree than in the Pteridophyta. Under the favourable conditions of the tropics the evolution of Pteridophytes progresses faster. The very regular formation of the cross-shaped bivalents in meiosis of the fern species points to some kind of mechanism, for example, localization of chiasmata, that prevents the formation of multivalents.

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OBSERVATIONS ON THE MODE OF REPRODUCTION AND THE EMBRYOLOGY OF POA PRATENSIS

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IN earlier publications (JULÉN, 1954, 1958 and 1960) data have been given about the effects in the first generation after X-ray treatment in *Poa pratensis* and the possible influence of the X-ray treatment on the reproductional mechanism of the species. The investigation presented here is a continuation of this work, and comprises embryological investigations of the mother clone, used by JULÉN, and selected plants in different generations of the X-rayed material, as well as observations on the seed setting behaviour as judged from observations of the progeny in generations up to the fourth after X-ray treatment. Some data are also given on the seed formation in crosses between apomictic and sexual plants and in F_1 after crossing two sexual plants.

I. X-RAY TREATMENT AND FIELD OBSERVATIONS ON DIFFERENT GENERATIONS

X-ray treatment was performed in 1950. Dry seed from a clone propagation of the commercial variety Fylking was given X-ray doses of 12,500—30,000 r. The number of seeds in each treatment was 1,000. Observations of the material were made in the first generation (X_1) in the field in 1951 and after vegetative cloning of the plants in 1952 and 1953. A scheme of the procedure followed, and the arrangement of plants of different generations for comparisons in the field is given in Fig. 1. Morphologically aberrant plants were found in the X-rayed material in a larger frequency than in the unirradiated control. In the

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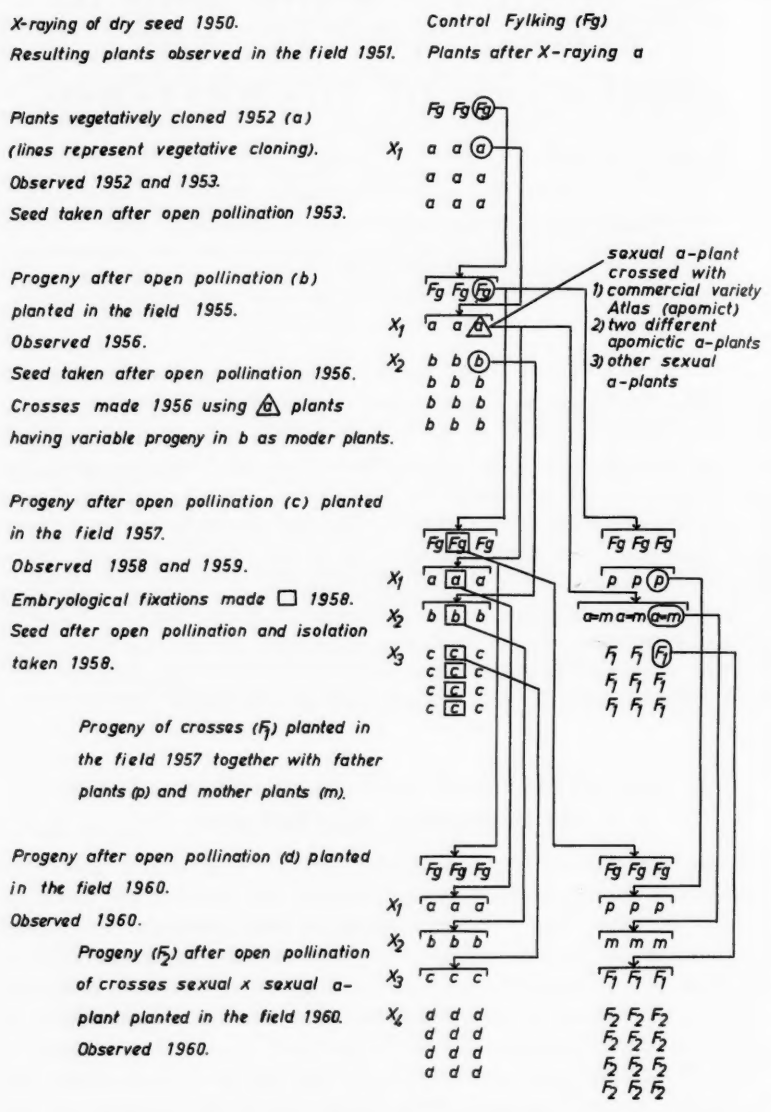

X-raying of dry seed 1950.
Resulting plants observed in the field 1951.
Control Fylking (Fg)
Plants after X-raying a
Plants vegetatively cloned 1952 (a)
(lines represent vegetative cloning).
Observed 1952 and 1953.
Seed taken after open pollination 1953.
Progeny after open pollination (b)
planted in the field 1955.
Observed 1956.
Seed taken after open pollination 1956.
Crosses made 1956 using (a) plants
having variable progeny in b as moder plants.
sexual a-plant
crossed with
1) commercial variety
Atlas (apomict)
2) two different
apomictic a-plants
3) other sexual
a-plants
Progeny after open pollination (c) planted
in the field 1957.
Observed 1958 and 1959.
Embryological fixations made □ 1958.
Seed after open pollination and isolation
taken 1958.
Progeny of crosses (F1) planted in
the field 1957 together with father
plants (p) and mother plants (m).
Progeny after open pollination (d) planted
in the field 1960.
Observed 1960.
Progeny (F2) after open pollination
of crosses sexual x sexual a-
plant planted in the field 1960.
Observed 1960.
X1
X2
X3
X4

Fig. 1. Procedure followed in planting and observation of the X-rayed plants and their progeny in different generations.

control material 2.0 per cent of the plants obtained were noted as aberrants. These may be assumed to have been formed by sexual seed formation — through fertilization of a reduced egg cell or through fertilization of an unreduced aposporous cell. The frequency of aberrants formed in the X-rayed material after different doses are given in Table 1 (data from JULÉN, 1954):

TABLE 1. *Frequency of aberrants and chimaeras in Poa pratensis after X-irradiation of dry seed.*

X-ray units:	0	12.500	15.000	17.500	20.000	25.000	30.000
Aberrants in per cent of surviving plants	2.0	3.8	4.9	11.6	14.2	14.1	no surviving plants
Chimaeras in per cent of surviving plants	0.13	3.24	3.06	5.98	8.33	8.28	

It is not possible to decide whether a non-chimaerical aberrant with a chromosome number close to that of the original clone is the result of sexual seed formation or due to an effect of the X-ray treatment. Most of the chimaera-formation may, however, be assumed to be due to changes caused by the irradiation, and the examples for illustrating the procedure in the investigation have therefore been chosen among plants found to be morphological chimaeras and with a chromosome number close to that of the mother clone. To avoid variations in the material due to twins (MÜNTZING, 1937) all twin seedlings were excluded.

The habitus of some of the plants noted as aberrants in the X_1-generation was very markedly changed. The changes affected characters as length and thickness of straw, length and breadth of leaves, length and breadth of the panicles, number of branches in the panicle, density of the tuft, the creeping capacity, the number of fertile sprouts, the colour of the leaves, the occurrence of anthocyanin in leaves and panicles, the time of emergence and the resistance to mildew and rust.

Seed was taken after open pollination of the X-rayed material in 1953. Observations on the X_2-generation were made in 1956 on material planted in 1955. Progenies of plants or parts of chimaerical plants morphologically different from the mother plant and morphologically similar to it were studied. In both those groups a considerable increase in the frequency of variable progenies and thus in the incidence of sexual seed formation was found compared to that expected from the behaviour of the control material (Table 2, cited from JULÉN, 1958).

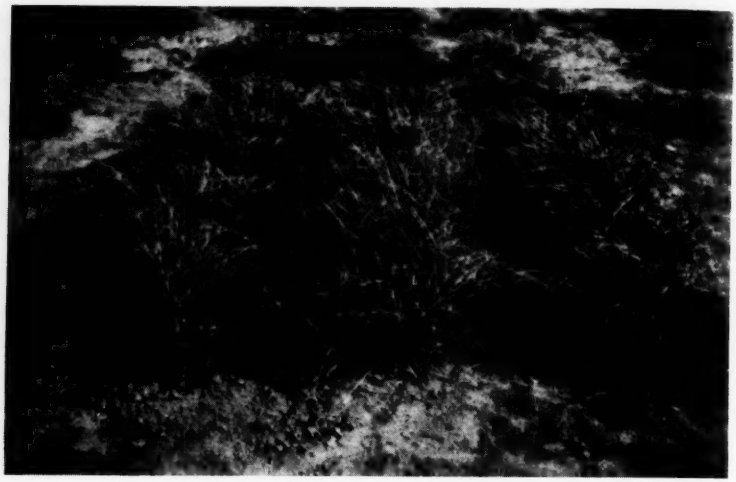


Fig. 2. Apomictically formed plant progeny.

Fig. 3. Apomictically formed plant progeny.

Fig. 4. Partially apomictically, partially sexually formed plant progeny.

Fig. 5. Sexually formed plant progeny.

TABLE 2. *Manner of seed formation in X_1-plants.*

	Total	Apom.	Part. sex.	Tot. sex.
Plants morphologically similar to the mother clone	110	81	22	7
Per cent		73.6	20.0	6.4
Plants morphologically different from the mother clone	176	83	61	32
Per cent		47.2	34.6	18.2

In the morphologically chimaerical plants the seed formation was sometimes sexual in all parts, sometimes apomictic in all parts, and in many cases sexual or partially sexual in one part, apomictic in another. It also happened that plants which were morphologically uniform were chimaerical with regard to seed formation.

It can not be stated with any certainty from a numerical point of view that the X-ray treatment has induced sexual seed formation. The frequency of variable progenies of the X_1-plants and the frequency of aberrant plants in the Fylking clone are not comparable entities, the observations being made in different years. The occurrence of chimaeras in much higher frequencies in X-rayed material than in the control makes it probable, however, that X-ray treatment induces the chimaera formation with regard to seed setting as well as in morphological characters.

In 1956 seed was harvested after open pollination of a number of the X_2-plants. Crosses (mass pollinations without emasculation) were also made. X_1-plants proved to have mainly sexual seed formation were used as mother plants in crosses with on one hand other sexual plants, on the other hand three different apomictic father plants, two of them from the X-rayed material, the third from the commercial variety Atlas. The X_2- and F_1-plants were observed in the field in 1958 and 1959, and seed after open pollination and isolation taken in 1958 from a number of X_2- and F_1-plants and from X_1-plants used as mother plants in crosses. The seed formation behaviour of plants — if apomictic or sexual — has been decided by putting 30 seeds per plant out for germination. The seedlings have been planted in boxes and later transplanted into individual pots, keeping the plants in the order in which germination had taken place. All twins were recorded and excluded from the investigation.

Preliminary observations on the uniformity or variability of the progenies were made in the greenhouse, and 12 plants per progeny chosen for further observation in the field, care being taken that equal pro-

portions of early and late germinating plants were kept for field planting. Observations in the field have been made by noting differences or similarities in habitus and development in the progenies and between mother and progeny plants, arranged side by side in the field (see Fig. 1). Measurements of straw-height, leaf-height and plant circumference and recordings of the shooting date have given somewhat more objective informations about the variability in the material.

In accordance with JULÉN's (1958) terminology progenies with 0—1 deviating plants have been noted as apomictically formed, those mother plants that have had 2—9 deviating plants have been termed partially sexual, while those with 10—12 deviating plants have been said to be sexual. Examples of apomictic, partially sexual and sexual progenies are given in Figs. 2—5.

II. MATERIAL FOR THE EMBRYOLOGICAL INVESTIGATIONS

Material for embryological investigations was fixed in the field in 1958. Spikelets from different parts of the panicle from 3 panicles per plant were fixed in MÜNTZING's modification of NAVASHIN's fluid. New fixations of the control material were made in spring 1960 on plants transplanted from the field to the greenhouse just before flowering. Whole panicles or parts of panicles were fixed at this time, care being taken to fix panicles in later stages of development than in the 1958 fixations. Meiosis starts first in the flowers at the top of the panicle and proceeds downwards. Inside the spikelets the lowest flower is the oldest. The largest number of favourable stages for embryological investigation were found when, in the middle part of the panicle, the lowest flowers of the spikelets had mature pollen, the upper flowers meiotic stages in the anthers. This was checked by orcein squash examination of the anthers. The spikelets — with or without glumes — were embedded in paraffin and sectioned at 10 μ. Iron hematoxylin was used for staining. The best results were received by prolonging the mordanting and staining times to 10—20 hours each. No counterstain was used. The slides were mounted in Canada balsam.

A total of 672 slides with 2,112 flowers was used in the embryological investigation. To give a quick, complete and detailed view of the behaviour of a plant, and for comparisons between plants, a graphical way of presenting the data was worked out and has been largely used (Figs. 11, 12). Free hand drawings, more or less schematical, from the

microscope, have been made in a large number of cases. Some of the most interesting are presented in this paper (Figs. 7, 8, 16, 23, 24. For explanations see the text).

III. RESULTS

1. Embryological observations in the Fylking clone

In the field the variety Fylking, which was used in this investigation, is very uniform, and the results of JULÉN (1954), where 2 per cent aberrant plants were found in the unirradiated control material confirmed the assumption that seed formation under field conditions in this variety was to a large extent apomictic. It was thought to be of interest to make clear the embryologic background in the original material as well as in some of the X-rayed material, that had shown a deviating behaviour with regard to seed formation. Fixations in control and X-rayed material were made in 1958. However, the Fylking material fixed was unfortunately at a too early stage of development, and new fixations had to be made in the spring of 1960. Because of the different times of fixation the investigations of the Fylking material and the rest of the embryological investigations made are not strictly comparable. Altogether 339 flowers from 9 panicles form the basis of the conclusions about the embryological behaviour in the Fylking clone. Although a very large variation was found in embryological behaviour in Fylking, some conclusions about the most common series of events leading to seed formation may be made from the relative frequency of cases (Fig. 6). The main features of development agree with earlier investigations on the embryology of *Poa pratensis* (*e.g.* TINNEY, 1940; ÅKERBERG, 1942).

Aposporous cells, most commonly only one in each flower, may appear very early, when the PMC is still in prophase. They are usually found in the chalazal part of nucellus, close to the EMC. (1) At a somewhat later stage of development, when meiosis starts in the PMC, the aposporous embryosac initials are most easily recognizable and can be found in a large number of the investigated flowers (2). As a rule the legitimate EMC produces only three megaspores, the second division in the micropylar dyad cell being started but never accomplished (3). The aposporous cell grows towards the center of nucellus and shows several vacuoli, which soon fuse into two big polar vacuoli giving the typical picture of the 1-nucleate ES (4). The chalazal one of the 3 megaspores grows and sometimes reaches the 1-nucleate ES-stage. The other

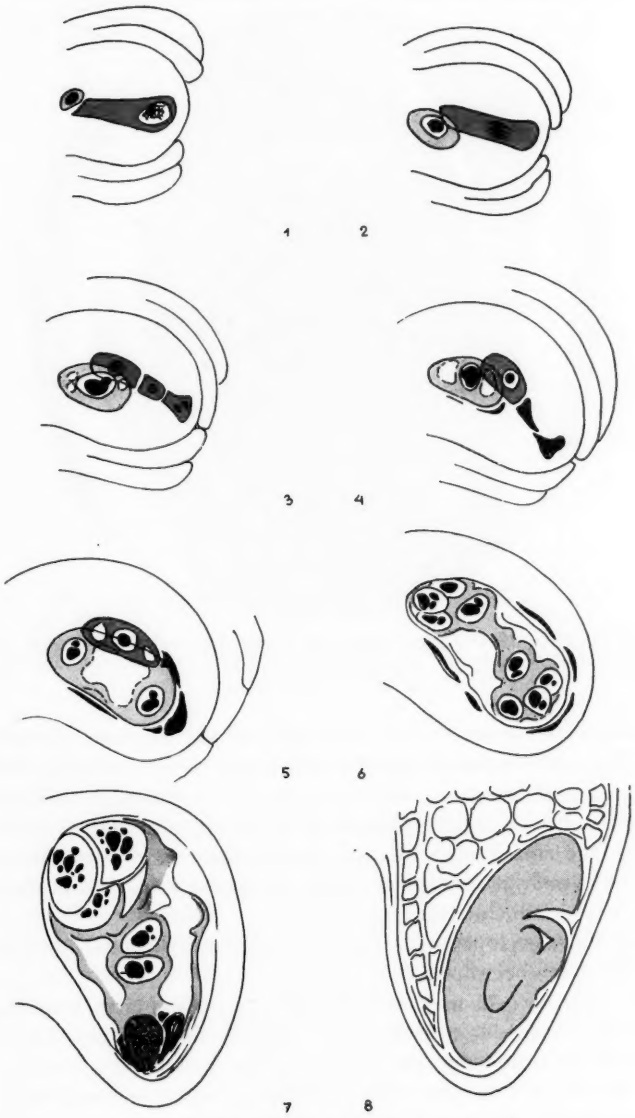


Fig. 6. Seed formation in the Fylking clone.

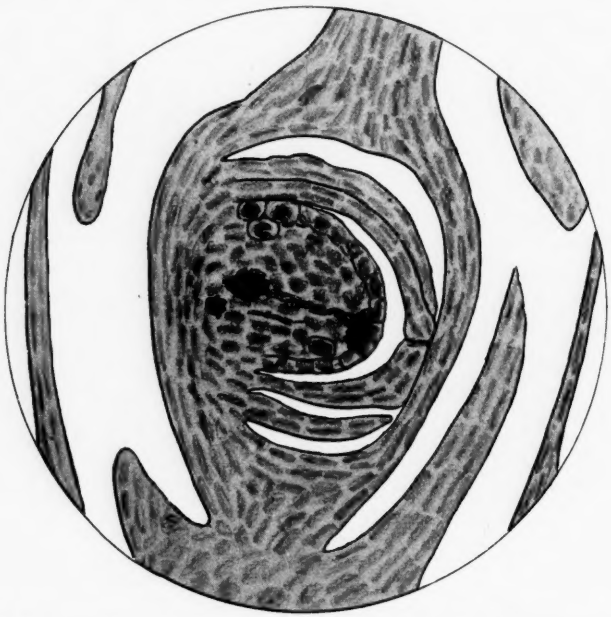


Fig. 7. Degeneration of both sexual and aposporous cells. Microspores were formed in the anthers.

megaspores degenerate (4—5). The aposporous embryosac initial grows towards micropyle and its nucleus undergoes three successive divisions, forming an eight nucleate embryosac (5—6). At this stage the legitimate megaspores are completely degenerated (6). In later stages, just before anthesis, the embryo has already been formed, while the two central nuclei have not yet fused. Very large antipodal cells rich in chromatic material are visible at this stage (7).

While this development is the most commonly encountered, several deviations have been found.

a) Aposporous cells may start appearing only at a later developmental stage and continue to appear until very late stages. More than one — sometimes several — aposporous embryosac initials may develop. 33 % of all investigated flowers with aposporous cells contained more than one aposporous embryosac initial. The position of the aposporous cell in nucellus may be more central or sometimes micropylar.

b) Aposporous cells may be lacking altogether in some flowers. Those

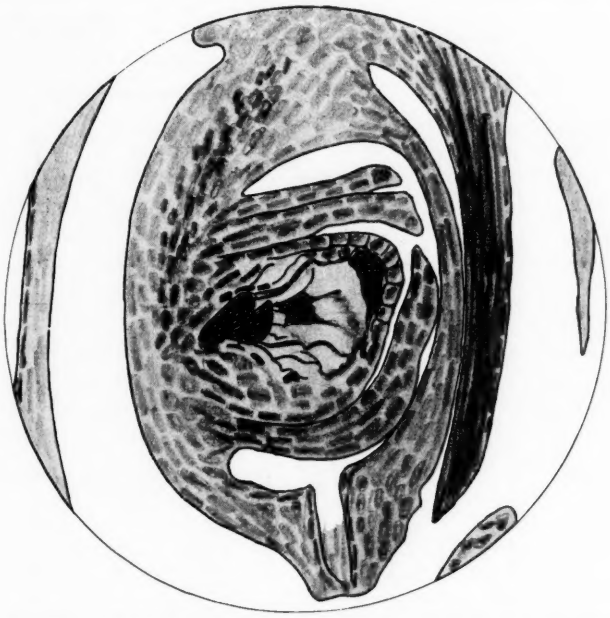


Fig. 8. Degeneration of the embryo-sac, the three dark spots representing egg apparatus, central nuclei and antipodal cells. The stage in the anthers was first pollen mitosis.

have been recorded as sexual. However, there is no certainty that aposporous cells may not appear in later stages, if the investigated flower has been fairly young.

c) Degeneration of aposporous cells may occur. Dark areas due to degenerated cells were sometimes seen in the chalazal part of nucellus, where aposporous cells normally arise, while the legitimate EMC was seen developing normally.

d) Degeneration of the EMC before meiosis may happen but is extremely rare (only one case has been found). Degeneration of all megaspores soon after meiosis was sometimes seen.

e) Degeneration of both sexual and aposporous cells occurred in some cases without any apparent reason. This degeneration occurred sometimes before, sometimes after embryosac formation (Figs. 7 and 8).

The male development is more regular, but more or less complete degeneration of some microsporangia was observed occasionally, and a few large polynucleate PMC:s were seen.

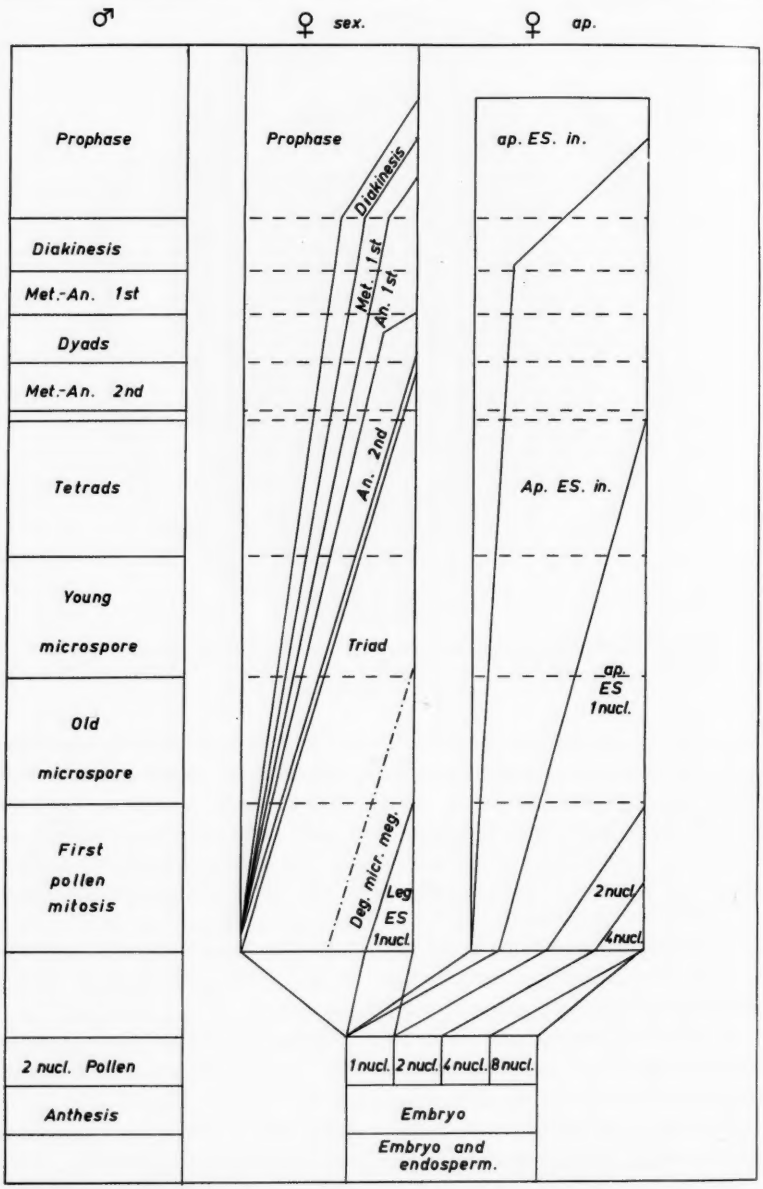


Fig. 9. The rate of development of the male side compared to that of the female legitimate and aposporous cells in the Fylking clone.

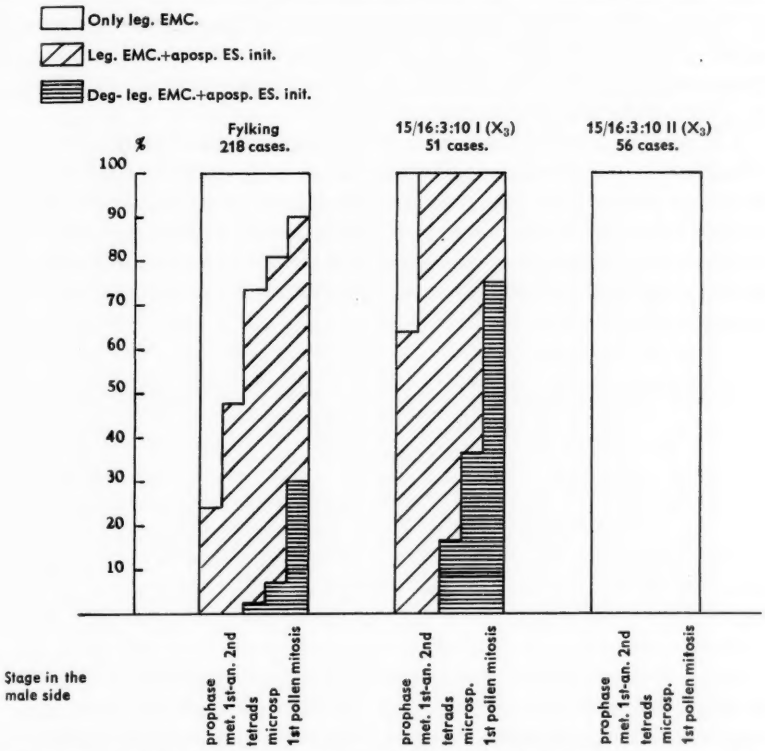


Fig. 10. The relative frequency of flowers in different stages with aposporous cells, and with degeneration of the legitimate female line in combination with occurrence of aposporous cells, in the Fylking clone and in two X_3 sister plants with strongly apomictic and strongly sexual behaviour respectively.

A comparison between the male and female rate of development is given in Fig. 9. It can be seen from the figure that there is no absolute correlation between the stages in the male and in the female side. The EMC may be found still in prophase when the microspores enter the first pollen mitosis. On the other hand the EMC may sometimes be found in a later stage of meiosis than the PMC. As a rule, however, meiosis starts and is accomplished earlier in the PMC. The aposporous embryosac initials start appearing in prophase stage (ap. ES. in.) and already while the PMC is still in late prophase big aposporous cells (Ap. ES. in.) may be found in nucellus. The aposporous cells may continue

to appear until very late stages, their chances of successful competition with the legitimate EMC probably being smaller the later they appear. Degeneration of the female side has been found only in stages later than tetrads in the male side.

It can be seen that aposporous and sexual cells exist together in nucellus for a considerable period. The chance of getting sexually formed embryosacs exists, increased by the possibility of degeneration of the aposporous cells. Table 3 and Fig. 10 show the relative frequency of flowers in different stages with aposporous cells, and with degeneration of the legitimate female line in combination with occurrence of aposporous cells.

TABLE 3. *Occurrence of aposporous cells in different stages of development in Fylking.*

Stage in the anthers	Number of invest. cases	Flowers with ap. cells, %	Ap. cells+deg. leg. cells, %
Prophase	55	23	—
Met. 1st—An. 2nd	23	48	—
Tetrads	38	74	3
Microspores	42	81	7
1st pollen mitosis	60	90	30

It can be seen that in a fairly large number of flowers (23 %) aposporous cells are present already at the time of prophase stage in the anthers in Fylking. During meiosis the frequency of cases with aposporous cells increases, and at the tetrad stage it is 74 per cent. The first cases of degeneration of the megaspores appear here. At the time of the first pollen mitosis 90 per cent of the flowers contain aposporous cells, and in 30 per cent of the flowers degeneration of the legitimate female line has occurred. In 30 per cent of the flowers we are thus certain that only the aposporous cells are capable of development. Even in those cases it may happen, however, that fertilization of the aposporous ES occurs, and non-maternal progeny is formed. In 10 per cent of the flowers no aposporous cells were found even at the stage of pollen mitosis. In half the number of those, aposporous initial cells had apparently been present but degenerated. In those flowers — 5 per cent of all flowers in the plant — sexual seed formation is apparently to be expected with greater probability, unless degeneration of the embryosac occurs. In the remaining 60 per cent of the flowers the possibility of apomictic seed formation exists, but the result of the competition be-

tween aposporous and legitimate cells is not yet clear. The number of aposporous cells in a flower and their stage and position in nucellus in relation to the legitimate EMC, may indicate a more or less pronounced probability for them to win the competition in the individual case.

In later stages of development than at the first pollen mitosis it is not possible to draw any reliable conclusions whether the aposporous or the sexually formed embryosac has won the competition, except by conclusions from the chromosome number, which is extremely difficult to decide in this material, especially with the staining method used for the embryological investigation. In observations of later developmental stages it was found that the egg cell in Fylking started to divide immediately after the sac was mature. This is in agreement with earlier findings (ÅKERBERG, 1942; NYGREN, 1951). However, only a small number of flowers in late stages have been investigated, and we know very little as yet about the variation in this respect in Fylking or in the other material investigated.

Comparisons were made between different parts of the same panicle and between different panicles, to see if the panicle was homogenous in regard to the possibilities for seed formation. As mentioned earlier, meiosis starts in the upper part of the panicle and proceeds downwards. If the difference in developmental stage is taken into consideration the degree of apomictic tendency seems to be uniform inside the panicle. The median part of the panicle shows less variation in different respects (number of flowers per spikelet, stage of development, proportion of flowers with aposporous embryosac initials) and has a smaller number of aberrations than the top and bottom of the panicle. The comparison between panicles, on the other hand, indicates a fairly large variability, both from the point of view of degree of apomixis expressed as the number of cases in which aposporous cells were found, and in regard to the position of the aposporous cells and the type and number of aberrations found.

The investigation of a single panicle received from a Fylking plant in the greenhouse in early spring indicates that the environmental conditions may have a definite influence on the balance between apomictic and sexual cells, a considerably larger percentage of flowers without aposporous ES initials being found during late developmental stages in this plant than in the other Fylking plants investigated. The results from this panicle are not included in the average values for Fylking given in the tables. Fig. 11 shows the number of flowers in different stages and their tendency to aposporous or sexual development in the

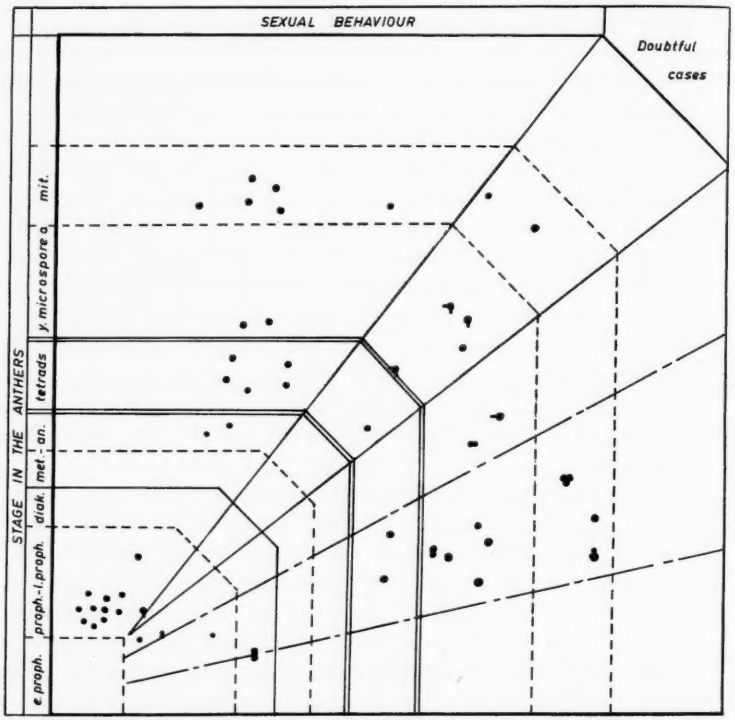


Fig. 11. The embryological behaviour of a Fylking plant flowering in the green-house in early spring.

In the graphs (11, 12, 14, 15, 17—22) each investigated flower in a plant is represented by a dot. Where no aposporous cells were found in the flower the dot is placed in the upper left sector of the graph. The further the development in the female line has gone without appearance of aposporous cells, the further to the left the dot is placed, indicating the greater chances for sexual seed formation to take place.

If aposporous cells have been found in a flower, the dot has been placed in the lower right sector of the graph, the size of the dot indicating the size of the aposporous cell and several dots together representing the appearance of multiple aposporous cells. The position of the dot — if near the lower margin of the graph or closer to the central sector — is decided by the relation between the aposporous and legitimate cells under development in the flower. A case where degeneration of the legitimate cell is taking place and apomictic seed formation may be expected with a high degree of certainty, is represented by a dot placed close to the lower margin of the graph, while a flower with a normally developed legitimate cell in an advanced stage compared to the stage of development of the aposporous cell, is represented by a dot near the central sector.

In the central part of the graph, finally, cases are represented where seed setting

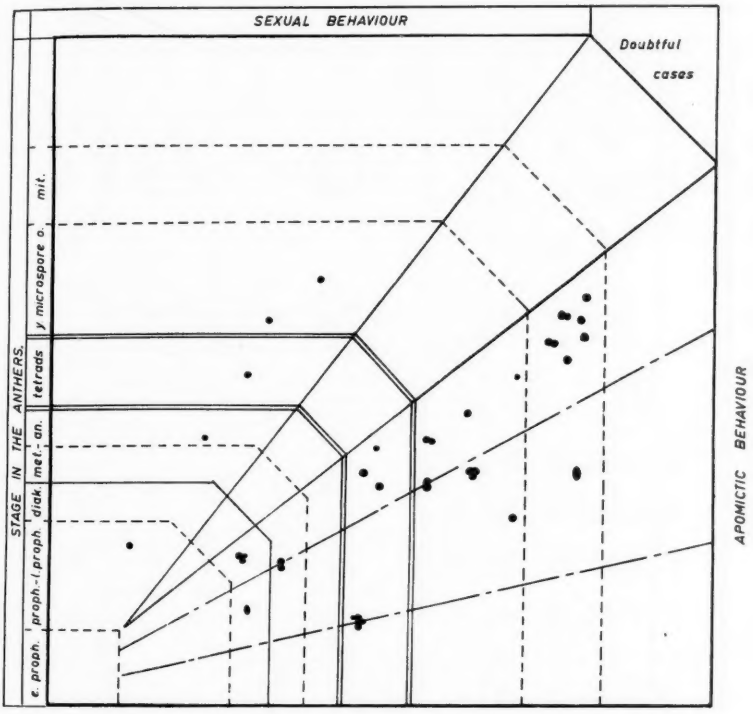


Fig. 12. The embryological behaviour of a Fylking plant flowering in the field.

plant fixed in the greenhouse, while Fig. 12 gives the development in Fylking in the field.

2. Observations on the morphology and embryology of X_1-plants found to have sexual seed formation and their progeny

From the seed harvested after open pollination of the vegetatively cloned X_1-plants, X_2-plants were planted out in the field and observed in 1956 together with again vegetatively cloned material of the X_1 mother plants. Seed was harvested from the X_2-plants in 1956 and the resulting

may not be expected to take place for the reason of degenerations or aberrations appearing in the flower. For all the flowers the dots are placed in the appropriate field according to the stage of development in the anthers given in the left margin of the graph.

Fig. 13. The reproductional behaviour in different generations in a family originating from an X-ray treated seed (125/85).

1 Dry seed X-ray treated 1951. A resulting plant, 125/85, found to be morphologically deviating from the mother clone Fylking.

2 X_1-plant, 125/85 vegetatively cloned into nine parts and found to be morphologically chimaerical, four parts being of one type, five of another.

3 X_1-plant, 125/85:2 vegetatively cloned for comparison with

4 its progeny in X_2 after open pollination. X_2 found to be morphologically variable, indicating that seed formation of the X_1-plant 125/85: 2 had taken place sexually.

5 X_1-plant, 125/85:6 vegetatively cloned for comparison with

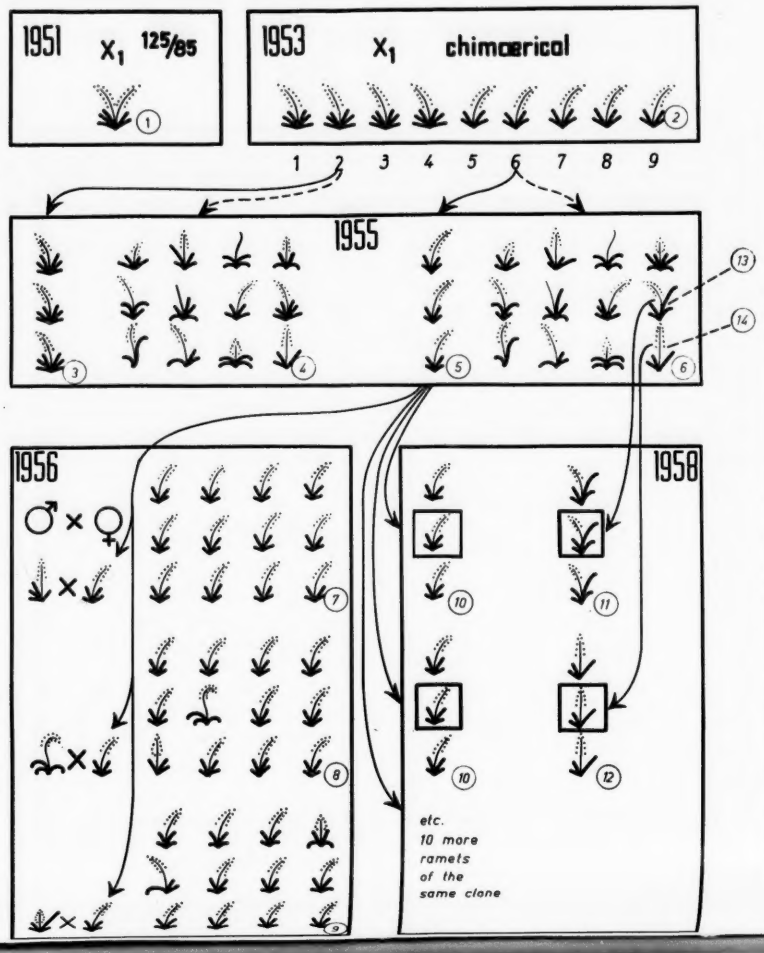
6 its progeny in X_2 after open pollination. X_2 found to be morphologically variable, indicating that seed formation of the X_1-plant 125/85:6 had taken place sexually.

7—9 X_1-plant, 125/85: 6 used as mother plant in attempts to crosses with three different apomictic father plants. Progeny in F_1 formed mainly apomictically. (No non-maternal plant in one cross, 2 out of 12 non-maternal F_1-plants in each of the two other crosses.)

10 X_1-plant, 125/85: 6 vegetatively cloned into 12×3 parts for comparison with progeny in X_2 and X_3. 12 parts embryologically investigated 1958. Competition between aposporous and sexual cells found during embryosac development. Progeny of one of the parts after open pollination in 1958 observed and found to be mainly non-maternal.

11—12 X_2-plants 125/85:6:11 and 125/85:6:12 vegetatively cloned into 3 parts for comparison with progeny in X_3. One part of each X_2-plant embryologically investigated. Results uncertain because of fixation at early stages in embryological development.

13 X_2-progeny after open pollination of the X_2-plant 125/85: 6: 11 morphologically uniform and maternal, embryologically uniform and apomictic, indi-



in X_2. One part of each X_2-plant embryologically investigated. Results uncertain because of fixation at early stages in embryological development.

13 X_3-progeny after open pollination of the X_2-plant 125/85: 6: 11 morphologically uniform and maternal, embryologically uniform and apomictic, indicating that seed formation in the X_2-plant had taken place apomictically.

14 X_3-progeny after open pollination of the X_2-plant 125/85:6:12 morphologically non-maternal and variable, embryologically variable, indicating that seed formation in the X_2-plant had taken place sexually.

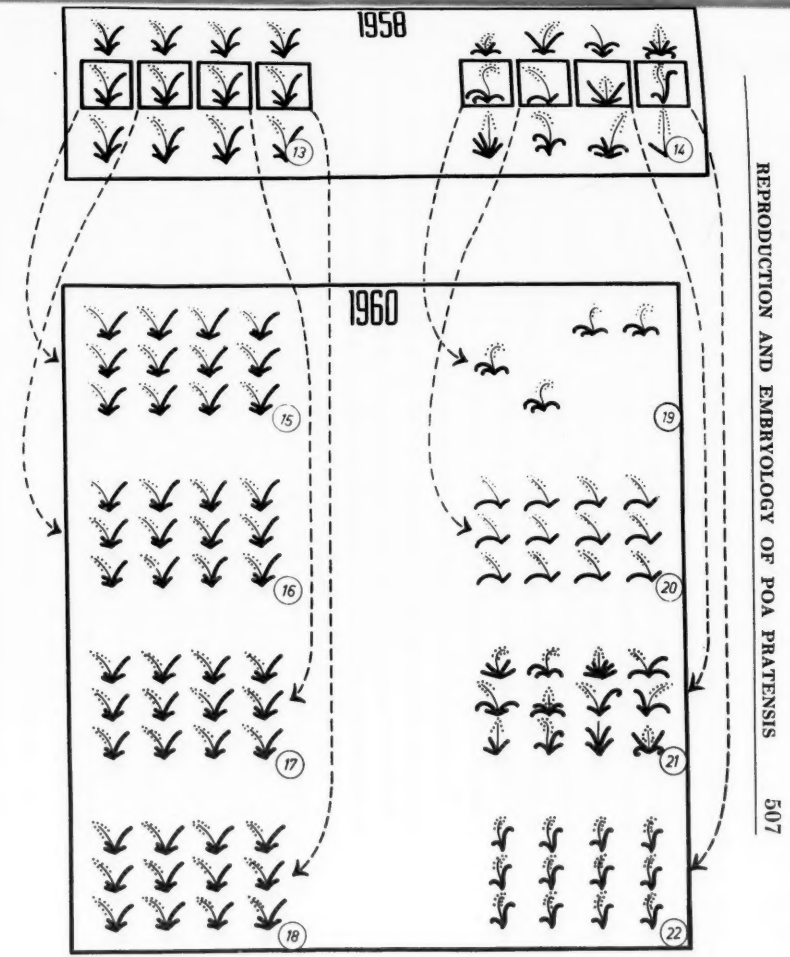
15—18 X_4-progeny after open pollination of the X_3-plant 125/85: 6: 11: 2, 125/85: 6: 11: 5, 125/85: 6: 11: 8 and 125/85: 6: 11: 11 morphologically uniform and maternal, confirming the results of the embryological investigation of the X_3-plants.

19 X_4-progeny after open pollination of the X_3-plant 125/85: 6: 12: 2 uniform and maternal, confirming the results of embryological investigation of the X_3-plant, which showed this plant to be apomictic with a high degree of degeneration of the ovaries, causing semi-sterility.

20 X_4-progeny after open pollination of the X_3-plant 125/85: 6: 12: 5 uniform and maternal. Embryological investigation of the X_3-plant showed this to be apomictic and have many aberrations.

21 X_4-progeny after open pollination of the X_3-plant 125/85: 6: 12: 8 non-maternal and variable, confirming the results of the embryological investigation of the X_3-plant, which showed this plant to be sexual.

22 X_4-progeny after open pollination of the X_3-plant 125/85:6:12:11 uniform and maternal. Competition between aposporous and sexual cells during embryological investigation of the X_3-plant.



→ veg. cloning
- - → seed progeny
□ embryological investigation

X_3-plants observed in the field in 1958 and 1959. The X_2-plants were vegetatively cloned and the X_1-plants again vegetatively cloned and placed beside each progeny family together with likewise again vegetatively cloned material of Fylking, to make possible a direct comparison between different generations (Fig. 1). Thus, the original plants from X-rayed seed had gone through three consecutive vegetative clonings before fixations for embryological investigations were made in 1958. To illustrate the investigations the results from one family originating from an X-ray-treated seed will be given.

The family chosen as an example of the seed formation and corresponding embryological behaviour originated from a seed treated with 12,500 r, no. 125/85 (Fig. 13). The plant was divided into nine parts, and found to be a chimaera morphologically, with two types, one part low and with very broad leaves, the other part morphologically fairly similar to Fylking but with a few days earlier shooting. Seed was harvested from one plant from each of the two types, and the X_3 generation observed. All plants in the progenies of the two X_1-plants were found to be non-maternal and deviating from all other plants in the progeny. The seed formation thus seemed to have been sexual in both the chimaerical parts.

Seed was harvested after open pollination of the progeny in X_3 of one of the X_1-plants, the one most similar to Fylking in morphology. Observation of the X_3-generation in the field in 1958 showed that two of the progenies were uniform and morphologically similar to the X_2-plant, four contained both plants similar to the mother plant and plants different from each other and the mother plant, and in six progenies, finally, all progeny plants were different from each other and from the mother plant. According to these observations we had thus had a segregation in seed formation into 2 apomictic, 4 partially sexual and 6 sexual plants in the X_2-generation, although because of the small number of progeny plants observed the classification is uncertain. In each X_3-progeny seed after open pollination was harvested from 4 plants, and the X_4-progeny was planted in the field in spring 1960. The observations on these plants may not be entirely reliable, as the plants have not yet flowered and the time of shooting and morphological characters of the panicles are valuable for classifying the plants. However, in most cases differences or similarities between plants are quite clear already at this stage of development, and some preliminary data may be given on the segregation (Table 4, family A).

It can first be stated that progeny in X_4 of X_3-plants formed apomic-

tically by X_2-plants giving uniform progeny without deviating plants (2 cases), was uniform with one possible exception — 2 plants in one of the 8 investigated progenies were found to be uncertainly deviating from the mother plant. Progeny of X_3-plants formed by apomixis in partially sexual X_2-plants seemed themselves to show some degree of sexuality. In not less than 8 cases out of 10 investigated in progenies from 4 different X_2-plants, some deviating plants were found, while in the other two cases apomixis seemed to have been complete in the X_3-plants. 5 cases from the same 4 X_2-progenies were investigated where the X_2-plant was sexually formed by a partially sexual X_2-plant. 2 of the 5 X_3-plants were found to be sexual while 3 appeared to have apomictic seed formation. Finally, of 24 X_3-plants, formed by sexual X_2-plants 5 were found to be apomictic, although in 2 of these cases there was some doubt about the complete uniformity of the progenies, 10 were found to be partially sexual and 9 sexual.

TABLE 4. *Manner of seed formation in X_3-plants as concluded from the morphology of X_4-plants in three families, A, B, C each derived from one X_1-plant with sexual seed formation.*

	X_3 formed sexually			X_3 formed apomictically		
Seed formation in X_3:	apom.	part. sex.	sex.	apom.	part. sex.	sex.
X_3-plants derived from sexual X_2-plant						
A	5	10	9			
B	7	6	8+2			
C	3	11	6			
	15	27	25			
%	23	40	37			
X_3-plants derived from partially sexual X_2-plant						
A	3	—	2	2	8	—
B	—	—	—	1	—	—
C	—	1	—	5	—	—
	3	1	2	8	8	—
X_3-plants derived from apomictic X_2-plant						
A				7	1	—
B				8	—	—
C				10	1	—
				25	2	—

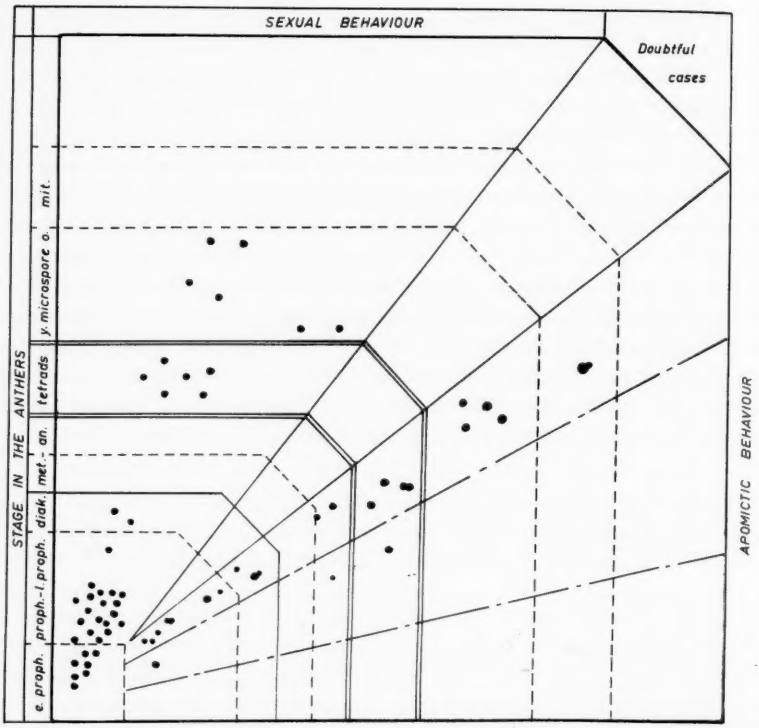


Fig. 14. The embryological behaviour of a clonal part of the X_1-plant 125/85: 6.

Investigations according to this pattern were made in 14 families each derived from one X_1-plant. The segregation in seed formation behaviour in X_2 was similar in all cases, no family being completely sexual and the number of apomictic plants in each group of 12 X_2-plants varying from 0 (in two cases) to 4 (in one case). The percentage of X_2-plants in the groups apomictic, partially sexual and sexual were 15, 25 and 60 respectively.

In X_3 the mode of reproduction of altogether 116 plants was observed, the X_3-plants originating from 3 of the X_2 families. The result of the progeny observation is shown in Table 4.

Embryological investigations were carried out in the family given as an example, originating from the plant 125/85, in 12 clone plants of the X_1-generation, in 2 X_2-plants and in 4 X_3-plants in each of the progenies of the two X_2-plants. Fig. 13 gives an indication about the results of the

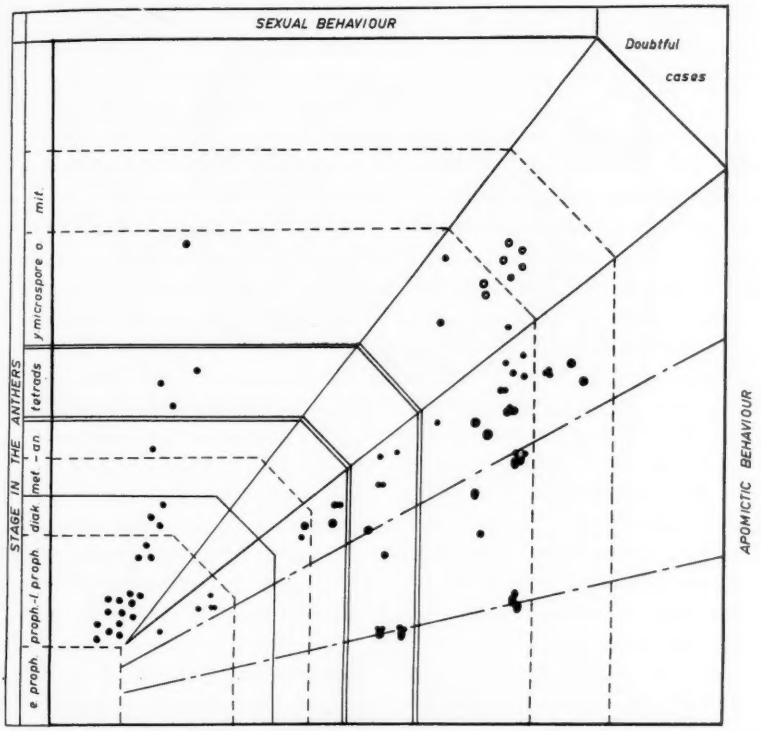


Fig. 15. he embryological behaviour of a clonal part of the X_1-plant 125/85: 6 (cf. Fig. 14). ○ = "round chalazal body".

investigation. The X_1-clone plants had been grown in the field under identical conditions, and spikelets had been fixed from all of them on the same day. Morphologically they seemed to be identical in the field, and the same was true at later observations of the same plants transplanted into pots in the greenhouse. The chromosome number of all the clone plants was $2n = \pm 74$.

Some possible differences were noticed between the plants when they were subjected to a closer examination in connection with the embryological investigations, however.

a) The stage of development of the plants with respect to flowering seemed to vary very considerably. This may, however, be entirely due to chance, panicles of somewhat different age having been chosen for fixation.

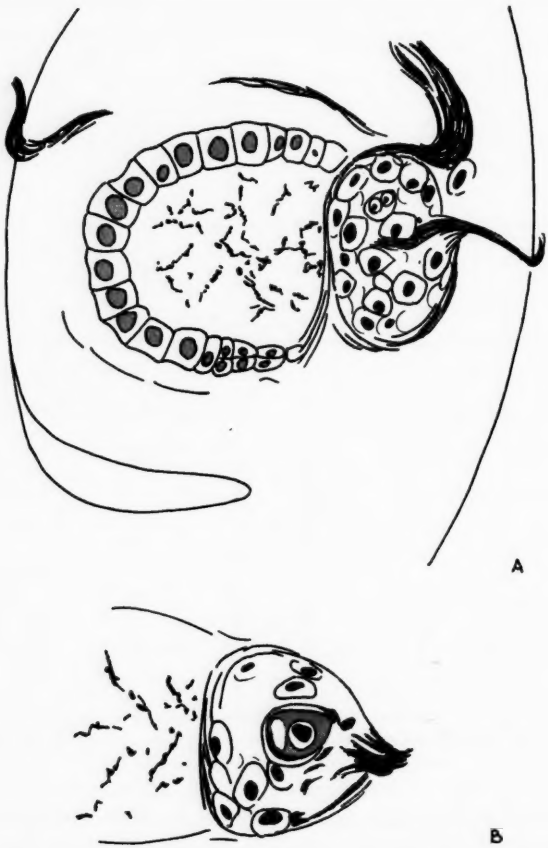


Fig. 16. A typical case of the aberration given the working name "round chalazal body". 16 B represents another section from the same flower where an aposporous-like cell is visible in the middle of the round body. The stage in the anthers was first pollen mitosis.

b) The number of flowers per spikelet, which in Fylking is quite constant, at least under identical environmental conditions, varied between different plants from 3.0 to 3.9, being fairly constant within one and the same plant. Care was taken when the fixations were made to chose flowers from all parts of the panicle, and the variation between different parts of the panicle, which was noticeable in Fylking, would not be expected to have had influence.

c) All the plants showed approximately the same type of competition between aposporous and legitimate embryosac development as Fylking. However, the variation between the clone plants is considerable. Some of the plants are presented in the graphs (Figs. 14, 15).

It can be seen from the graphs that the most obvious difference between the plants is in the relative frequency of "aberrations". The cases noted as aberrations are mostly of a type given the working name "round chalazal body" (Fig. 16). Alterations in ovaries of *Poa* may be due to insect damages (ANDERSEN, 1927), but so far there is no evidence that this is the case in our material. The characteristic picture is that of a cavity inside the epidermic layer of nucellus. Small darkly stained dots, similar to chromatic material are scattered in this cavity. From the chalazal part a round body, clearly differentiated from the surrounding tissue, is projecting towards the nucellar cavity. In some cases cells similar to aposporous embryosac initials were seen in this body (Fig. 16 B). However, it is hardly probable that seed can develop in flowers with this aberration. Seed setting may thus be decreased in plants showing this feature. The number of such aberrations varies from none in one plant, where 61 flowers were investigated to 10 in a plant, where 66 flowers were investigated. In two plants the aberrations started to appear already at early developmental stages, in most cases aberrations were not found until after meiosis in the male side. The variation in respect to relative number of apomictic cases in a plant also seems to be somewhat greater in the clone plants than in Fylking.

The over all picture in all the plants is, as was earlier mentioned, that of a balanced competition between aposporous and legitimate embryosac initials during embryosac development, a behaviour very similar to that found in Fylking. Unfortunately no fixations were made in later stages of development. From the earlier mentioned morphological examination of X_2-plants, we know that all the 12 daughter plants observed 1956 of this X_1-plant, showing a balanced competition between aposporous and sexual cells during embryosac development, were apparently formed in a sexual way, if by fertilization of a haploid or diploid nucleus we do not know in this case, as the chromosome numbers have not yet been decided.

In this connection, however, another family may be mentioned, in which chromosome counts have been made. The family has its origin in another X_1-plant, found to be chimaerical at the first observation. One of its parts was very similar to Fylking morphologically, and was also mainly apomictic like Fylking. Two parts deviated morphologic-

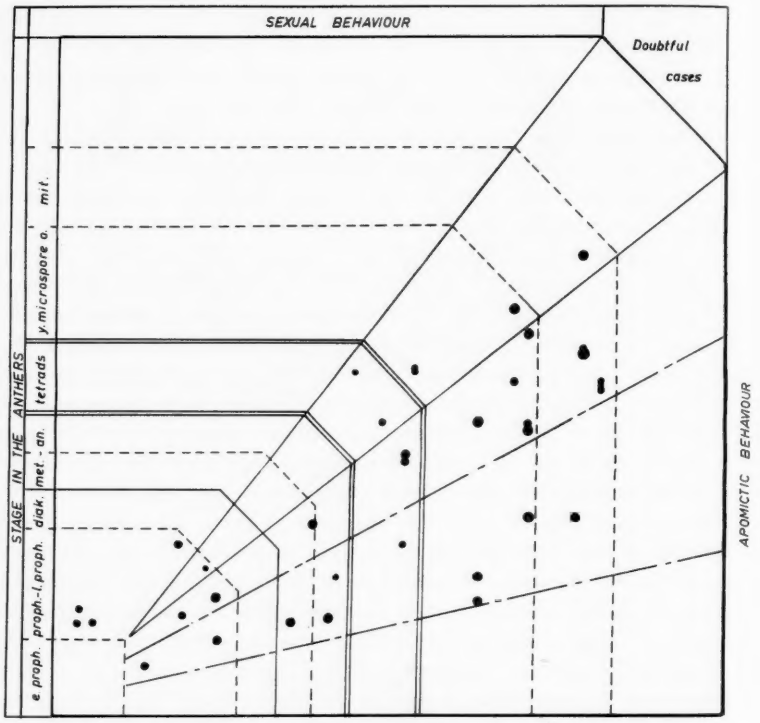


Fig. 17. The embryological behaviour of X_a plant 125/85: 6: 11 I.

ally from Fylking. One of those parts had apomictic seed formation, the other was partially sexual, as judged from the morphological variability of the progeny. The chromosome number of the X_1-plant was ± 71, that is, quite similar to that of Fylking. The plant cannot have been formed through parthenogenetic development of a reduced gamete, in which case the chromosome number would have been half that of Fylking (± 37) or through fertilization of a diploid aposporous cell, in which case it would have been 50 % higher than in Fylking, or ± 111. The possibility of seed formation after fertilization of a normal reduced gamete is unlikely, because the X_1-plant was a chimaera, and especially since one of the parts of the chimaera was apparently identical to Fylking both in morphology and manner of seed formation.

The chromosome numbers of the X_2-plants formed by the sexual X_1-

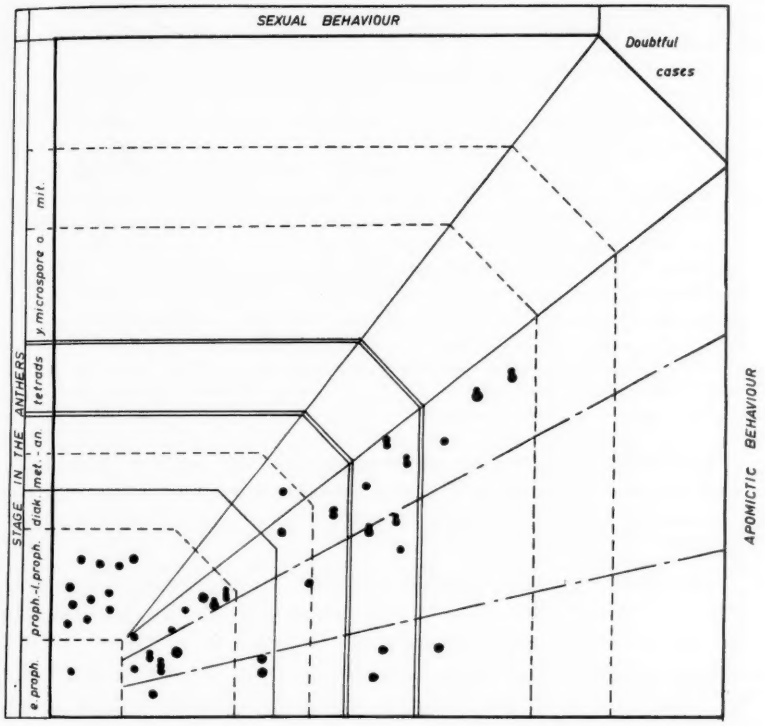


Fig. 18. The embryological behaviour of the X_3 plant 125/85: 6: 11 III.

plant were counted, and their manner of seed formation decided through progeny observation. The following list gives the chromosome numbers, possible way of formation of the embryo, and seed formation manner of the plant itself. The plants are arranged according to chromosome number.

The earlier mentioned plant, which was embryologically investigated, 125/85: 6, was also used as a mother plant in crosses with apomictic father plants in 1956. It was then found to behave as an apomict in one cross and to be partially sexual but with a pronounced apomictic tendency in two crosses. Seed after open pollination was taken in 1958 (the same year as fixations for embryologic investigations were made). The progeny of the plant was then found to be partly variable, partly

Plant nr. in X_2	Chr. number of the X_2-plants	Manner of fertilization in the X_1-plant	Seed formation of the inv. X_2-plant
1	± 71	Fertilization of reduced gamete	apomictic
2	71	„ „ „ „	partially sexual
3	74	„ „ „ „	apomictic
4	74	„ „ „ „	sexual
5	75	„ „ „ „	apomictic
6	75	„ „ „ „	apomictic
7	75	„ „ „ „	partially sexual
8	75	„ „ „ „	sexual
9	112	Fertilization of dipl. ap. cell	partially sexual
10	112	„ „ „ „ „	sexual
11	114	„ „ „ „ „	sexual
12	150	Fertilization of dipl. ap. cell by unreduced male nucleus	sexual

identical to the mother plant, which must then be classified as partially sexual. Its apomictic tendency is also confirmed by the fact that a large frequency of twins were found (6 out of 26) when the seed was germinated.

It should perhaps be stressed that there is no question about the correctness of the classification of the progeny from seed harvested in 1953 as completely sexually formed. Repeated observations of the daughter plants in X_2 during 1956—60 have all given the result that they were different from each other and from the mother plant. In the X_2 generation of 125/85: 6 two plants were investigated embryologically. However, both of these were in early developmental stages and no definite conclusions can be drawn about their seed setting behaviour. In both of them aposporous cells were found and also a few flowers without sexual cells in stages later than meiosis. There is thus indications of the same kind of balanced competition during embryosac development as in Fylking and in the X_1-plants. However, the later development in the two plants has apparently gone in different directions. It was found that the two X_2-plants had quite different manners of seed formation according to observations of the morphology of the X_3-plants and according to embryological investigation of the X_3-plants. The seed setting behaviour of the X_2-plants was in its turn confirmed by progeny observation. One of the X_2-plants, 125/85: 6: 11 gave morphologically uniform progeny. The four daughter plants investigated embryologically showed that uniformity was found also in this respect, as the following descriptions and Figs. 17 and 18 show.

125/85: 6: 11 I. Early development of aposporous cells, which appeared and grew in a more central position in nucellus than usual. No flower without aposporous cells found among 30 investigated. Several flowers with multiple (2—3) aposporous cells found. Degeneration of all megaspores found in ¾ of the stages when microspores had been formed in the anthers. Very large, abnormal PMC:s and pollen grains found. 2 twin seedlings found among 28 germinated seeds after open pollination, 4 among 27 after isolation. Progeny uniform.

125/85: 6: 11 II. Early development of aposporous cells in central position. Three cases of flowers with only sexual cells found among 34 investigated. Many cases of multiple (3—4) aposporous cells found. Degeneration of all megaspores found in about half the number of cases where microspores were found in the anthers. Abnormalities noted in the anthers. In later stages 6 cases found showing "round chalazal body" in nucellus. 2 twin seedlings found among 27 after open pollination, 4 among 29 after isolation. Progeny uniform.

125/85: 6: 11 III. Early development of aposporous embryosac initials in central or micropylar position. No case of completely sexual behaviour found among 41 investigated cases. Multiple aposporous cells (2—3) found in many cases. Degeneration of all megaspores found in microspore or later stages. (Few late developmental stages investigated.) Abnormalities found in the anthers. 2 twin seedlings found among 24 germinated after open pollination, 2 among 23 after isolation. Progeny uniform.

125/85: 6: 11 IV. Spikelets fixed in younger stages than in plants I—III. All flowers except two were found to be in premeiotic stages. However, already in stages before meiosis aposporous cells were found in some instances. One flower found with "round chalazal body". 2 twin seedlings found among 23 germinated after open pollination, 3 among 27 after isolation.

The embryological behaviour of the apomictically formed progeny plants should be identical to that of the mother. If we compare the mother plant 125/85: 6: 11 to the daughter plants I—IV we find that there is nothing to oppose this expectation. The mother plant was fixed in an earlier stage (younger panicles) than any of the daughter plants with the possible exception of IV. No case with later stages than tetrads in the anthers was found in the mother plant. Aposporous cells were found to appear already in prophase stages in similar numbers to those in comparable stages in the daughter plants. No case of the "round chalazal body" was encountered in the mother plant, but in most cases those did not appear in the daughter plants until microspores were formed. The results indicate that this X_2-plant has a considerably more pronounced apomictic tendency than either the original material, Fylking, or the mother plant. The possibility of sexual seed formation in a small number of cases is not excluded according to the embryological investigations, however.

The other investigated X_2-plant, 125/85: 6: 12, gave a morphologically very variable progeny, with all the 12 daughter plants different

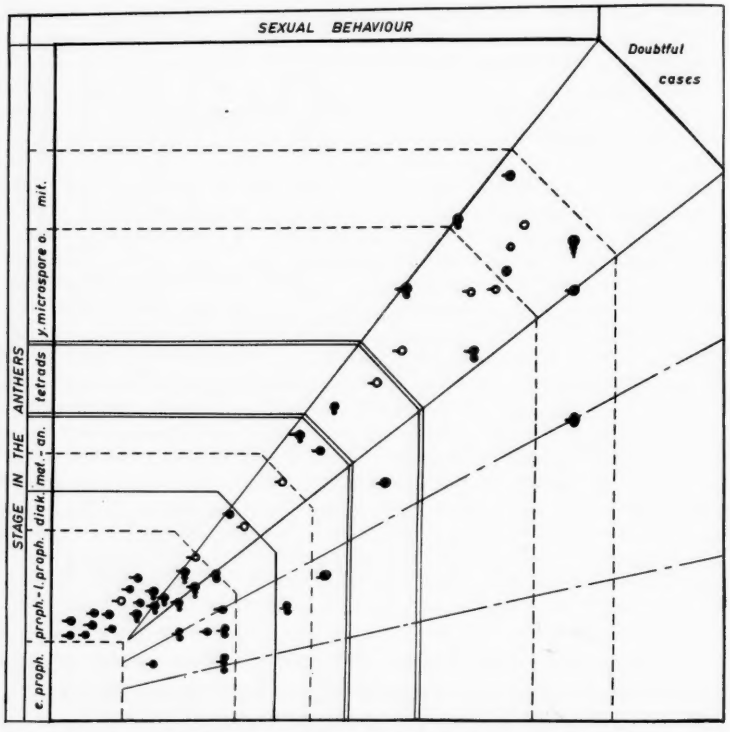


Fig. 19. The embryological behaviour of the X_a plant 125/85: 6: 12 II. —=degeneration of anthers, ○=round chalazal body.

from each other and from the mother plant. In the four daughter plants investigated embryologically a large variation was also found, as is shown by the following description and Figs. 19—20.

125/85: 6: 12 I. Complete degeneration of nucellus appeared very often and already in early stages, and in older flowers degeneration was found in almost all flowers. Aposporous embryosac initials were found in a few flowers. Partial degeneration of the anthers was found in many cases. Seed setting was very poor both after open pollination and after isolation. 33 seeds were produced in 5 panicles after open pollination, 6 after isolation. Only seven of the former germinated, three of them being twins, and none of the seeds produced after isolation germinated. The four plants after open pollination were very similar to each other, but were all very weak and died before transplantation into the field.

125/85: 6: 12 II. Early development of aposporous embryosac initials. Several cases

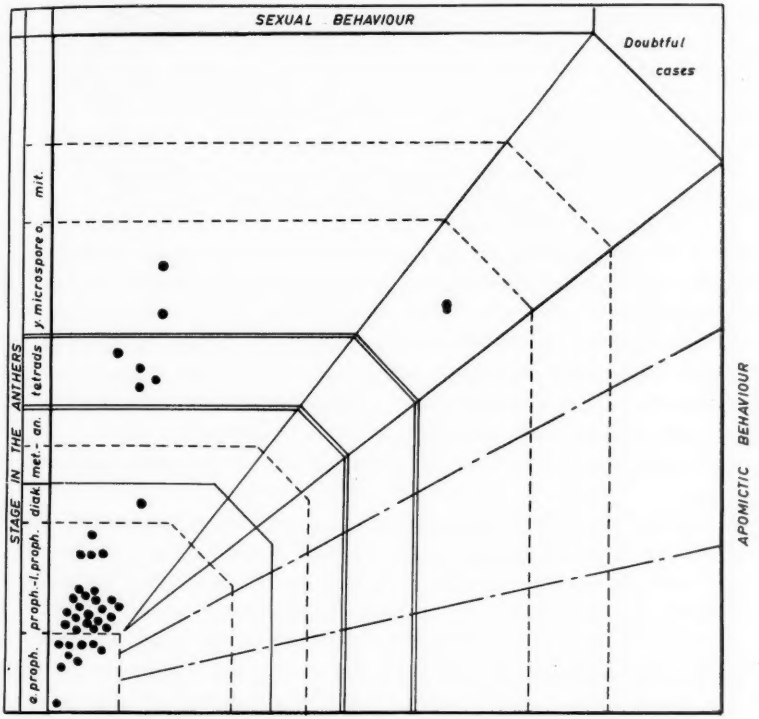


Fig. 20. The embryological behaviour of the X_a plant 125/85: 6: 12 III.

of "round chalazal body" found. Multiple aposporous cells (2—3) found in many cases. Most of the anthers completely degenerated (Fig. 19). Seed setting was fairly good after open pollination. 4 twin seedlings were found among 27 germinated seeds. The progeny after open pollination was uniform. Of 30 seeds produced after isolation only 8 germinated. Two of these were twins, the rest seemed to be morphologically uniform in the seedling stage but were never transplanted to the field.

125/85: 6: 12 III. Only sexual cases (6 in postmeiotic stages out of 40) were found (Fig. 20). Seed setting seemed to have taken place in a sexual way according to the morphological variation among the progeny plants. No twins were found among 28 germinated seedlings. Two of the seedlings were albinos (only 4 were found in the *Poa* material germinated in 1959, altogether about 10,000 seedlings).

125/85: 6: 12 IV. Aposporous cells started to appear at the early prophase stage of the anthers, and were found in co-existence with the legitimate EMC all through the stages. No case of degeneration of the legitimate EMC was found. However, no slide with flowers in stages later than microspores was investigated. A few cases with

multiple aposporous cells were noted. Seed setting was good. No twins were found either among 27 germinated seeds after open pollination or 23 after isolation. Progeny both after open pollination and isolation was quite uniform, and morphologically identical to the mother plant, indicating that the result of the competition between legitimate and aposporous cells is development of the later.

Summing up the results of the morphological and embryological investigations of the plant 125/85: 6 and its progeny we find that

1) The plant in 1958 shows embryologically a similar behaviour to that of the original clone Fylking, a balanced competition taking place between aposporous and sexual cells in nucellus during embryosac development.

2) The seed formation of the plant has been at least mainly sexual in 1953, when seed was taken after open pollination. Seed formation has been mainly apomictic when attempts were made to cross it with one father plant, partially sexual in crossing attempts with two other plants 1956. Seed formation was partially sexual after open pollination in 1958.

3) In the sexually formed X_2-progeny after open pollination 1953 segregation into apomictic, partially sexual and sexual plants took place, two plants being mainly apomictic, four partially sexual and six mainly sexual as decided by progeny observation.

4) One sexual and one apomictic (according to progeny observation) X_3-plant was investigated embryologically in 1958 together with their X_3-progenies. The results confirmed the classification of one plant as apomictic, the other as sexual, variation being found also embryologically in the progeny of the latter.

5) Morphological observations of the progeny of the embryologically investigated X_3-plants confirmed the results from the embryological investigation, showing that the X_4-progenies of all the plants termed as apomictic in the uniform X_3-progeny were uniform and thus apomictically formed.

In the sexually formed progeny one X_3-plant was found to be semisterile and apomictic in agreement with the embryological observations, one was found to be apomictic and almost sterile after isolation confirming the concept of *Poa pratensis* being pseudogamous. In the embryologic investigation this plant had been found to be mainly apomictic and almost pollen sterile. In a third X_3-plant only sexual cells were found at the embryologic observation, and the X_4-progeny was in agreement with this very variable. The fourth X_3-plant embryologically had a similar behaviour to the X_1-plant 125/85: 6 and to Fylking, with ba-

lanced competition between aposporous and sexual cells during embryo-sac development. Progeny observations showed that seed formation had taken place apomictically.

3. Observations on the morphology and embryology of X_1-plants found to have partially sexual or apomictic seed formation and their progeny

Seed was harvested also from X_2-plants deviating from the mother plants in families where the mother plant had had partially sexual or mainly apomictic seed formation in 1953. Out of 68 sexually formed daughter plants from 27 partially sexual X_1-plants 19 were found to have uniform progeny and can be designated as apomictic, 12 were partially sexual and 37 sexual. 13 sexually formed daughter plants from 13 mainly apomictic X_1-plants were investigated as to their seed formation behaviour through observation of the progeny. 5 of them were found to be apomictic, 3 partially sexual and 5 sexual.

As an example in this group a family originating from a seed which received an X-ray dose of 15,000 r may be chosen. The plant, 15/16, was found to be a chimaera with two different morphological types, one fairly similar to Fylking, the other very low with dark green leaf colour. Both parts had the same chromosome number as Fylking. Seed after open pollination was taken from one clone plant belonging to each of the mentioned types.

15/16: 6, the lowgrowing, dark plant, was found to have mainly apomictic seed formation, only one progeny plant deviating morphologically from the mother. The deviating X_2-plant had the chromosome number ± 75, and was apparently formed through fertilization of a legitimate, reduced egg cell. This X_2-plant had sexual seed formation.

15/16: 3, the X_1-plant similar to Fylking was found to have partially sexual seed formation, six of the twelve investigated progeny plants being morphologically identical to the mother plant, six clearly deviating. One of the sexually formed X_2-plants was partially sexual and two were sexual. Three were not investigated.

Fixations for embryological investigations were made in 1958 from the X_1-plant 15/16: 3, from one of the sexually formed X_2-plants, which had shown sexual behaviour in the progeny test, and from four of its progeny plants in X_3. The X_1-plant was fixed in too young a stage for any conclusions to be drawn. In the X_2-plant 58 flowers were investigated, 22 of these in stages later than meiosis. In no case was any apo-

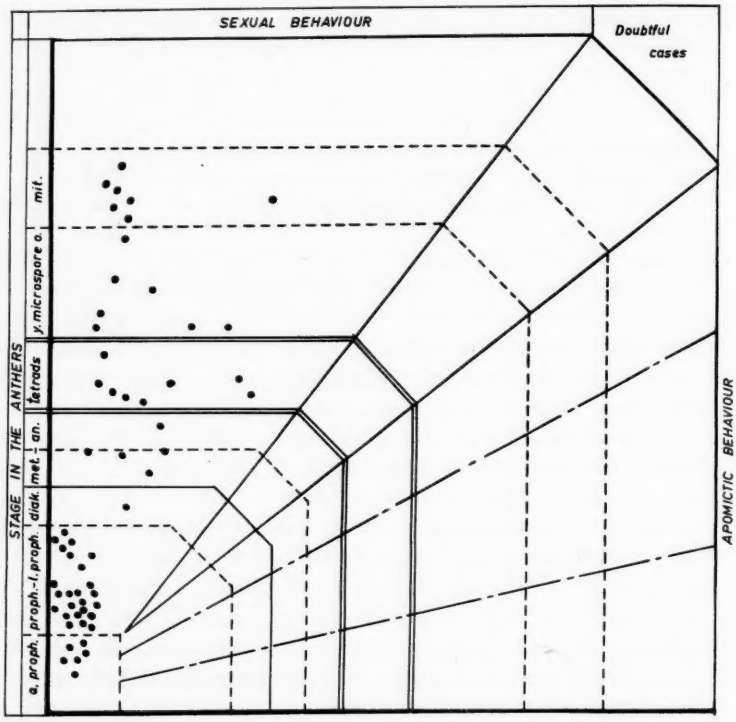


Fig. 21. The embryological behaviour of the X_2 plant 15/16: 3: 10 showing strong sexual tendency.

sporous cell found. The sexual tendency seems to be very strong in this plant (Fig. 21).

The same is true about one of the progeny plants in X_3, which shows no aposporous cell development in 56 investigated cases, 30 of these in stages later than meiosis (Fig. 10). Another investigated daughter plant (Figs. 22 and 10), however, has a very pronounced apomictic behaviour embryologically. 51 cases were investigated. The aposporous cells were very numerous (up to ten in the same nucellus) and big already in early developmental stages of the flowers. They were found everywhere in nucellus, in chalazal, micropylar and central positions. The EMC seemed to suffer from the competition with the aposporous embryosac initials already in early stages of its development, it was deformed and com-

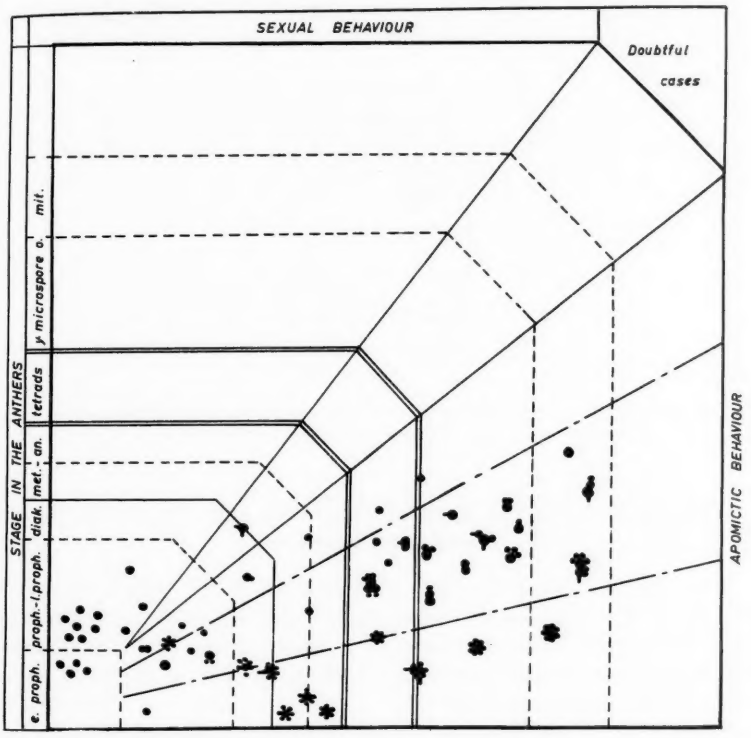


Fig. 22. The embryological behaviour of the X_s plant 15/16: 3: 10 I showing strong apomictic tendency.

pressed by the aposporous cells. Nevertheless it underwent meiosis and produced three megaspores. These were often compressed and looked very poor. In most of the cases they degenerated in later stages, while one or more aposporous embryosacs were growing.

In this case very extreme types have been found, the alternation between sexual and apomictic seed formation between the generations being very striking, and widely different types being found in the progeny of one and the same mother plant, with regard to reproductional behaviour.

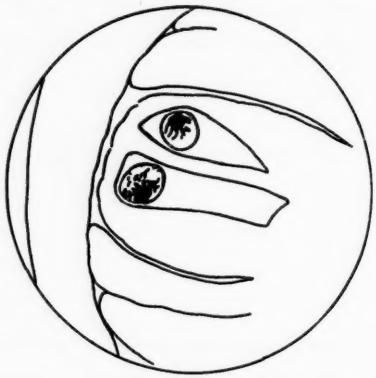


Fig. 23. Two embryo-sac mother cells both in prophase stage developing in the same nucellus and separated by a single layer of nucellar cells.

4. Observations on F_1 progeny in crosses between apomictic and sexual plants

Attempts were made in 1956 to cross 38 different X_1-plants, which had shown sexual tendency after open pollination in 1953, with different apomictic plants as father plants. The result of one of these attempts was mentioned in the description of the behaviour of plants used for embryological investigations.

In 1953 13 of the 38 plants had been partially sexual, while 25 had completely sexual seed setting. In 1956, however, only 12 of the plants behaved as sexuals in all crosses. Of the plants that had been completely sexual in 1953, no plant behaved as an apomict in all crosses 1956, but 9 of them produced only maternal progeny in at least one cross. The plant that showed the most pronounced apomictic tendency gave only maternal progeny in two cross combinations tried, and was partially sexual in the third. Of the 13 plants which had been partially sexual 1953 one behaved as a sexual in all crosses, one was partially sexual in all combinations, while 5 of the plants gave only maternal progeny in all attempts to crossing, the rest having varying degrees of sexual or apomictic seed formation in the crossing attempts.

To test if the behaviour of the mother plants as sexual in 1953 after open pollination was a temporary effect of the irradiation, seed was taken after open pollination from the same mother plants in 1958. The observations on the progeny plants is not yet finished. It can be said

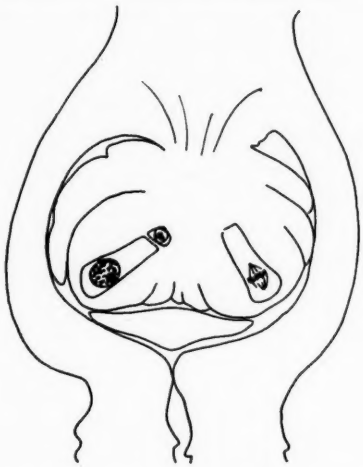


Fig. 24. Two ovules fused in the chalazal part of the nucellus, each with an EMC, one in the diakinesis stage and with an aposporous embryosac initial beside it, one in metaphase first.

with certainty, however, that the progeny has in no case been formed entirely apomicticaly, but partial sexuality has been found in several instances also where the plant had been designed as completely sexual in 1953.

A high frequency of twin seedlings may be taken as an indication that an apomictic tendency is to be found in the mother plant. At least one of the seedlings is generally formed by an aposporous embryosac (two sexual embryosac mother cells may be found in a flower, but apparently only in exceptional cases, Figs. 23 and 24). To how large an extent apomictic seed formation takes place in the mother plant cannot be decided by observation of the twin frequency, however, and a plant that does not produce twins may or may not have sexual seed formation. In 14 of the 25 plants that had been noted as completely sexual in 1953, twin seedlings were found after open pollination 1958, which indicates that aposporous cells were formed in the mother plant. Five of those plants, however, produced no maternal progeny either after open pollination in any of the years or after crossing.

The embryology of the plants was not investigated, except for the plants mentioned earlier, but the results seem to indicate that a competition between aposporous and sexual cells is the most probable be-

haviour to expect in most cases. The variation in the progeny may be caused not only by true hybridization, but also through the production of haploids, triploids and tetraploids. In the cases where twins were produced, but all progeny plants in all instances were aberrant, it is possible that only one of the mechanisms in apomixis, the production of aposporous cells, functioned, while the other, division of the egg cell before fertilization, did not take place, the resulting progeny being a mixture of sexually formed diploids, triploids and perhaps tetraploids.

IV. DISCUSSION

1. General remarks

The results of the embryological and morphological investigations give a picture of competition on different levels as the main feature in the development of plants and populations of *Poa pratensis*. The most commonly encountered picture during embryologic development is that of a balanced competition between apomictic and sexual cells, a competition which apparently can be shifted in favour of one or the other type of cells by different agents. There exist a series of biotypes, each with a balance between sexual and apomictic development, where the equilibrium is different for each biotype, and where each biotype has a definite maximum range of variation in regard to the extent to which seed formation may be influenced. The results of the competition between different biotypes in a population may be widely different in different environments.

MÜNTZING (1940) gives the concept of apomixis in *Poa* as "due to a rather delicate genetic balance", and draws the conclusion from available results that "this balance may be upset in various ways, by crosses with other types or merely by a quantitative change in chromosome number either in a plus or a minus direction".

The investigation presented here confirms the concept of apomixis as due to a very delicate balance, and throws some light over the intricate processes and balance systems governing seed formation in the species. A number of interrelated factors influence the seed setting process in different ways and at different levels, the complex interplay of all the factors causing plants of a certain biotype to give a certain proportion of sexually or apomictically formed seed under certain environmental conditions. The extent to which environmental factors can change the seed setting behaviour may vary from biotype to biotype depending on the stability of the interacting factors.

A complex genetic system governing the interplay of factors leading to apomictic or sexual seed setting may be expected. The clarification of the genetic background of apomixis in *Poa pratensis* will however be extremely difficult if not entirely impossible. It is known from earlier investigations in the species, and demonstrated to some extent in this material, that the chromosome number in *Poa pratensis* is extremely variable. Viable plants have been found with chromosome numbers from 18 (KIELLANDER, 1942; ÅKERBERG and BINGEFORS, 1953) to 147 (LÖVE, 1952) and 150 (this investigation). The plant with 147 chromosomes found by LÖVE (1952) was apomictic, while the one with 150 chromosomes in our material was sexual. The basic number in the species is 7, but not only multiples of this number, but apparently almost every possible aneuploid number from a certain level has been found. Chromosome counts in the species are indeed very difficult and especially the very high numbers counted are probably not exact. It would, however, be expected that at least some grouping around the multiples of seven should occur. This does not seem to be the case, however. Apparently plants may exist, produce viable pollen and produce seed sexually or apomictically with any chromosome number above 2n=45—50.

An analysis of the inheritance of genetic factors carried by chromosomes in *Poa pratensis* seems to meet with difficulties almost impossible to overcome. A number of facts may be brought out to justify this statement.

1. The chromosome number is high. Only in two exceptional cases has the number 2n=18 been found (KIELLANDER, 1942; ÅKERBERG and BINGEFORS, 1953), and these plants were not typical of the species *Poa pratensis* but very similar to *Poa trivialis*. The inheritance of apomixis may only for the reason of the polyploidy in normal *Poa pratensis* be expected to be very complex.

2. Meiosis is very often irregular (MÜNTZING, 1932; RANCKEN, 1934; FLOVIK, 1938; ÅKERBERG, 1942) and leads to production of functioning gametes with variable chromosome numbers. The possibility of getting valid information about the inheritance of apomixis from crosses is then very small. The irregularities in meiosis may cause losses or additions of chromosomes in the gametes on both the male and the female side. The possibility of the variations in this case being due to the existence of B-chromosomes may perhaps not be excluded.

3. A variable progeny may not always or at least not entirely be the result of fertilization of legitimate embryosacs. Legitimate, haploid em-

bryosacs may develop parthenogenetically, or aposporous embryosacs may become fertilized and give rise to aberrant progeny. The former case demonstrates the functioning of one of the processes in apomixis, the ability of parthenogenetic development of an embryosac, the other case shows that aposporous cells are being formed in the mother plants. Moreover, fertilization may occur with unreduced or reduced pollen grains, which is indicated by the presence of a plant with the chromosome number ± 150 in an X_3-progeny.

Applying and extending ÅKERBERG's (1942) scheme for seed formation we get the following possibilities for the family in which the mentioned plant with $2n = \pm 150$ was found:

	Gametophyte formation	Sporophyte formation	Expected chromosome number	Morphology in comparison with mother plant
Vegetative cell	Apospory	Diplo-parthenogenesis	Diploid ±74 (not found)	Maternal
	Apospory	Fertilization with haploid pollen	Triploid ±111 (0—3 cases?)	Aberrant
	Apospory	Fertilization with diploid pollen	Tetraploid ±148 (1 case)	Aberrant
Archesporial cell	Reduction division	Fertilization with haploid pollen	Diploid ±74 (8 cases)	Aberrant
	Reduction division	Fertilization with diploid pollen	Triploid ±111 (0—3 cases?)	Aberrant
	Reduction division	Haplo-parthenogenesis	Haploid ±37 (not found)	Aberrant

ÅKERBERG (1942) regards the possibility of fertilization by an unreduced pollen grain as less probable than that of fertilization of an unreduced egg nucleus with reduced pollen, for the production of triploids in his material. The reason for this was, that in crosses between plants with different chromosome numbers the results agreed with the latter possibility, and that no giant pollen grains had been observed.

The tetraploid plant found in our material seems, however, to be explained most easily by the assumption that an unreduced egg nucleus was fertilized by an unreduced pollen grain. Giant pollen grains were also met with in several of the investigated plants grown in the same field.

The result of the chromosome number investigation in the mentioned

family and of the embryological investigations shows that morphological examination of a plant progeny in reality tells us very little about the mother plant. Because of the occurrence of the tetraploid progeny plant we know that one part of the apomictic mechanism — the formation of unreduced gametes — functions in the actual mother plant, although, according to progeny observation it was completely sexual. We do not know for certain how the triploids had been formed. Theoretically the fertilization of a reduced egg nucleus by an unreduced pollen grain may in some instances take place more easily than the opposite occurrence. That would happen if the supply of unreduced pollen was good, and if, in a plant, aposporously formed egg cells start dividing before pollination is possible, while the division of the egg nucleus in sexually formed embryosacs is delayed. If twins are produced or not in such a case would depend on the relative vigour of the two competing embryos.

The embryological investigations demonstrate even more clearly that only morphological examination of the progeny, or even a combination of progeny observation and chromosome counting, does not give a reliable picture of the degree of apomixis in the plant. To give a complete picture of the behaviour of a plant from the seed formation point of view, morphological progeny observation, chromosome number control of every plant in the progeny as well as of the mother plant and a thorough embryological investigation of the mother plant comprising a series of developmental stages would be necessary. Because of the lability of the seed formation system investigations would have to be carried out in different environments. Such investigations are planned.

The present investigation gives some indications of the instability in the seed formation system and the places where it may be expected to be influenced, but may be regarded only as an indicator that further and more exact investigations are needed both regarding the influence of X-raying on *Poa pratensis* and regarding the embryological processes in the species and their variation under different environmental conditions.

There are two major processes responsible for apomictic seed formation, the presence of both of which are prerequisites for apomictic seed formation to occur. The stability of those processes, but also the development in intermediary stages, decide the result of the seed formation process. The first of those two processes is the ability of a plant to form embryosacs with the unreduced chromosome number, which in *Poa pratensis* takes place through formation of aposporous embryosac ini-

tials. The second is the division of the egg cell without fertilization. There are surely gradations in the factors, influencing these two processes, however, and it is thought most adequate to discuss the possibilities of genetical or environmental influence on the chain of events leading to seed formation in chronological order, although the competition at some points in the chain may be of more decisive importance than at others.

2. Competition at formation of aposporous embryosac initials

The number of aposporous cells formed may be influenced. Plants have been found in the embryological investigation where no aposporous embryosac initials apparently were formed under the prevailing environmental conditions. We do not know, however, whether the concerned plants are really completely sexual under all conditions. We know in one case that genes for aposporous cell formation might be present in the plant. The mentioned plant gave rise to a number of daughter plants after open pollination, one of which formed an extremely high number of aposporous cells, while another had no formation of aposporous cells. In other investigated plants varying numbers of aposporous embryosac initials were found. In some cases aposporous cells were formed in the majority of the flowers, but only one or seldom two per flower, in other cases many aposporous cells per flower were found.

The time for the appearance of the aposporous cells also varied very much from plant to plant and also from flower to flower in the same plant. The time when development starts may be expected to have a decisive influence on the possibilities for the aposporous cell to compete with the ones sexually formed. It can be seen e.g. in the graphs over the development of Fylking that small aposporous embryosac initials were found in stages as late as when microspores were formed on the male side (Fig. 12).

3. Competition during development of the aposporous cells

The factors mentioned under 2. may have influence on the ability of the aposporous cells to compete during development. The rate of development of aposporous and sexual cells may be different, and the position of the aposporous embryosac initials may have influence. It is to be expected that a more central position in nucellus is more favourable from a nutritional point of view. The number of aposporous embryosac

initials formed may be important from the point of view of competition for nutrition and for space.

The regularity or irregularity of meiosis in the legitimate EMC may have influence on its ability to compete successfully. Irregularities in meiosis may be the reason for the degeneration of all megaspores simultaneously, which was sometimes observed. As a rule the chalazal megaspore survived the rest and degenerated at a later stage. The reason for this degeneration is obscure, as well as the degeneration of both sexual and aposporous cells which was sometimes observed. Degeneration of only the aposporous cells was also observed in several instances. Degeneration may be expected to be influenced by genetical or environmental factors. An indication of the latter was that degeneration of cells — aposporous or sexual or both — was more often found in the distal parts of a panicle than in the middle region.

Degeneration of the megaspores does not, as a rule, seem to be the result of an actual encroachment of the aposporous cell upon the legitimate ones or the opposite, but degeneration of one or the other takes place without any obvious reason. Neither is the appearance of aposporous cells caused by the degeneration of the legitimate cells. In most cases the aposporous cells appear long before the degeneration of the legitimate cells, and in many cases legitimate and aposporous cells exist together in nucellus for a very long time, and probably both form embryos in some cases.

4. Competition at fertilization and during embryo development

It has been demonstrated in several instances in earlier investigations that embryo development in *Poa* occurs at an early stage, when the flowers have not yet opened and anthesis has not begun (TINNEY, 1940; ÅKERBERG, 1942, 1943, and others).

In our material unfortunately only a small number of slides in this stage of development have been investigated. Those investigated all concerned the Fylking clone. The results of the cited investigations were confirmed — embryo development was found to start before pollination had been possible. However, we do not know much about the variations in this respect in the material. The reason for the non-agreement between embryological observation and the manner of seed formation as judged from the morphology of progeny plants may be differences in the capacity of the aposporous egg cell to divide at an early stage. Some of the plants which embryologically showed a balanced competition between aposporous and sexual cells were, according to

progeny observation, mainly apomictic, others were sexual. The latter may have had a delayed embryo development making fertilization of the aposporous egg nucleus possible. The chromosome number has not been counted and the assumption can unfortunately not be verified. It is clear from earlier investigations that the two factors, formation of aposporous or diplosporous cells with the unreduced chromosome number, and division of the egg cell without fertilization are independent of each other. Several examples are cited by GUSTAFSSON (1946).

If both haploid and diploid egg cells have the capacity of early division, haploids may be produced by development of the legitimate egg cell in cases where the legitimate cell for some reason — absence of aposporous cells, favourable time for appearance and rate of development, or degeneration of the aposporous cell — can compete successfully. Provided that the central nucleus is fertilized a haploid plant will develop. A number of such cases have been cited in the literature from *Poa pratensis* and *Poa alpina* (MÜNTZING, 1940; ÅKERBERG, 1939, 1942; HÅKANSSON, 1943, 1944). The frequency of haploids produced may vary with the mother plant (HÅKANSSON, 1943). The same author mentions that the frequency of haploids in the progeny may be increased if fertilization is delayed. We have here the possibility of an influence of the father plant or of the interaction between father and mother plant on the manner of seed formation of the mother plant in crosses. A larger frequency of haploids might be expected when pollen tube growth is slow. The genotype of the father plants may thus in this case have the ability to influence the manner of seed formation in the mother plant. This is however not detectable without chromosome counting of the progeny. Several other possible ways of an influence of the father plant exist, however.

In the attempts to cross one mother plant with several different father plants it was found that the seed setting of the mother plant had in some instances taken place apomictically, all progeny plants being maternal, while in other cases the progeny of the same mother plant was variable. Although the number of progeny plants observed in the field was too small — 12 per progeny — to allow any conclusions about minor variations in the level of apomixis or sexuality, the difference in numbers of maternal plants were in several cases so pronounced that there seems to be no doubt about the difference in the main course of seed formation. This difference is apparently attributable in some way to the father plant. It was found that most of the mother plants had the ability to produce aposporous embryosacs, as indicated by the oc-

currence of twins in their progenies, but that their progeny after open pollination in two different years was mainly deviating from the mother plant. Isolation in paper bags may have an influence on the manner of seed formation, but it seems unlikely that this factor should be responsible in this case, as all plants were isolated, but only some showed differences in seed formation. Pollen fertility was good in all the father plants, and in no one of the crosses the same father plant caused all of the mother plants to behave apomictically, although apomixis was more pronounced in crossing attempts with one of the father plants.

Possible ways of seed formation. (For explanations see page 534.)

Leg. ES	Ap. ES	Embryos formed	Morphology of progeny in comparison with the mother plant
1. early division of egg cell	early division of egg cell	haploid and/or diploid aposp.	aberrant or maternal
2. early division of egg cell	pollinated by reduced pollen	haploid and/or triploid	aberrant
3. early division of egg cell	pollinated by unreduced pollen	haploid and/or tetraploid	aberrant
4. early division of egg cell	degeneration	haploid	aberrant
5. pollinated by reduced pollen	early division of egg cell	diploid sex. and/or diploid aposp.	aberrant or maternal
6. pollinated by reduced pollen	pollinated by reduced pollen	diploid sex. and/or triploid	aberrant
7. pollinated by reduced pollen	pollinated by unreduced pollen	diploid sex. and/or tetraploid	aberrant
8. pollinated by reduced pollen	degeneration	diploid sex.	aberrant
9. pollinated by unreduced pollen	early division of egg cell	triploid and/or dipl. aposp.	aberrant or maternal
10. pollinated by unreduced pollen	pollinated by reduced pollen	triploid and/or triploid	aberrant
11. pollinated by unreduced pollen	pollinated by unreduced pollen	triploid and/or tetraploid	aberrant
12. pollinated by unreduced pollen	degeneration	triploid	aberrant
13. degeneration	early division of egg cell	diploid aposp.	maternal
14. degeneration	pollinated by reduced pollen	triploid	aberrant
15. degeneration	pollinated by unreduced pollen	tetraploid	aberrant

The influence of the father plants may take place in several different ways. As only observations of the morphology of the progeny plants have been made, we do not know in which way the deviating plants have been formed, and we do not know what possibilities for seed setting existed in the mother plants. The scheme on page 533 shows the different possibilities for seed formation that exist in individual flowers.

It must be kept in mind that more than one of those possibilities may be realised in the same plant. It can be seen from the scheme that aberrant plants may be produced in several different ways, and the term "sexual" applied to plants having given aberrant progeny is not quite correct. The progeny of one plant may be made up of not less than four different types of aberrant plants — haploids, diploids, triploids and tetraploids — not counting the possibilities of irregular meiosis leading to the production of fertilized or non-fertilized aneuploids. Only one type of plants, the sexually formed diploids may be said to be true hybrids between the father plant and the mother plant, and because of the manner of crossing in our investigation (without emasculation) selfings may have taken place, even more reducing the number of true hybrids.

It can be seen from the scheme, that in six cases (4, 8, 12, 13, 14, 15) where degeneration of one or the other type of cell occurs, there is no competition between different embryos. However, the relative number of those kinds of embryos being formed in a plant may be influenced by the pollen. This is especially true about the relation between triploids and tetraploids, which must be influenced by the amount of pollen of the different types formed. MÜNTZING (1940) mentions a case where in *Poa alpina* under certain conditions only reduced pollen grains are formed, under other environmental conditions both reduced and unreduced. This possibility may exist also in *Poa pratensis* and will have influence on the relative numbers of progeny plants of different kinds formed by the mother plant.

The rate of germination and growth of the pollen tube from different father plants in one and the same mother plant may be different. MÜNTZING (1940) investigated progeny of a polyhaploid (2n=36) plant of *Poa pratensis*, and found the chromosome numbers and the morphology of the progeny plants after open pollination to be extremely variable. After isolation the chromosome number was constant and the same as that in the mother plant, but the progeny was morphologically variable, showing that seed setting had taken place after fertilization of a reduced gamet. The haploid plant's own pollen was evidently un-

able to compete with the pollen of surrounding biotypes. On isolation no other pollen was available and self-fertilization took place. In an embryosac where the egg nucleus starts to divide early it would be necessary, to enable fertilization, that the pollen tube had time to grow down the style before divisions started. Rate of growth of pollen tubes may have influence on the relation between types 2: 6: 10 and 3: 7: 11 respectively, and differences in growth rate between haploid and diploid pollen will influence the relation between sexually formed diploids, triploids and tetraploids in the progeny.

The mentioned cases only have an influence on the relative numbers of aberrant progeny of different kinds formed by the mother plant. The relation between maternal and aberrant progeny plants may be thought to be influenced in at least two different ways. If both aposporous and sexual embryosacs are present in nucellus in approximately the same stage of development, the early fertilization of the sexual egg nucleus may cause it to start divisions earlier and get ahead in the competition between two embryos under development in the same nucellus. Another possibility is perhaps more probable. A competition may be assumed to take place between different embryos simultaneously developing in the same nucellus. An apomictically formed embryo may in some cases be more vital than one formed after fertilization of a legitimate ES with the pollen from a certain father plant, leading to mainly apomictic seed formation of the mother plant in certain crossing attempts, while the combination of the genome of the same mother plant with that of another father plant may result in embryos having a better vitality than those apomictically formed, the result being a higher frequency of aberrant progeny. The pollen may also influence the possibilities for competition in different plants through the role it plays in fertilization of the central nucleus. Although the species apparently has a very great tolerance to chromosome number differences between endosperm and embryo, there may very well exist differences in compatibility between endosperms and embryos of different constitutions.

The competition between embryos with different genotypes may be extended to the time of germination. In a mature seed one seedling may be fully developed, the other only partially (ANDERSEN, 1927). Especially in seeds kept for some time before germination the weaker seedling might be expected to have smaller chance of surviving. ÅKERBERG (1942) mentions that in one case the frequency of twins in a 3-year-old seed lot was lower than that in the newly harvested, although this was not confirmed in a later test.

ÅKERBERG (1939) also mentions cases where the father plant seemed to have an influence on the ratio of aberrant plants in crosses between *Poa alpina* and *P. pratensis*. Further investigations (ÅKERBERG, 1942) showed that zygote selection was the more probable explanation, the hybrid with *Poa alpina* germinating more rapidly than pure *pratensis*. If plants from the first seedlings were selected a higher frequency of hybrids would be found than when all seedlings were planted out. Under field conditions such a competition between seedlings with different genetical constitution would influence the final composition of a stand.

5. Competition in the field

The final stage in the competition at different levels, competition of plants in the field is really not inside the scope of this investigation, but will be touched upon very briefly. It has been mentioned several times in this paper that the behaviour of different plants from the seed formation point of view may be different under different environmental conditions. The same is true about the vitality of the plants. CLAUSEN and co-workers have discussed these problems in a large number of publications (e.g. CLAUSEN, 1954, 1959; CLAUSEN, HIESY and NOBS, 1959).

The variation brought out in the material after sexual seed formation is extremely large. Plants may be found superior or inferior to the original material both in general vitality as judged by spreading capacity and leaf production, and in seed setting capacity. Plants with different degrees of apomixis or sexuality seem to appear in all those groups. The possibility for new apomictic, superior types of *Poa pratensis*, formed through segregation after crossing, to compete successfully in a population, seem to be great. Also the possibilities for variation in the species through spontaneous mutations should perhaps not be underestimated. From the breeder's point of view lines with a strong apomictic tendency are desirable since superior genotypes can be kept stable and seed production of apomictic varieties can take place without distance isolation to prevent intercrossing. However, it is also desirable that variation can be induced for selection of new lines with special characteristics. The possibility of selecting or making crosses in localities where the balance mechanism of a certain variety is shifted somewhat in favour of sexual seed production exists (NYGREN, 1953). The possibility also exists, to bring about chimaera formation in morpho-

logical characters and also in the functioning of the seed setting mechanism through treatment with ionizing radiation.

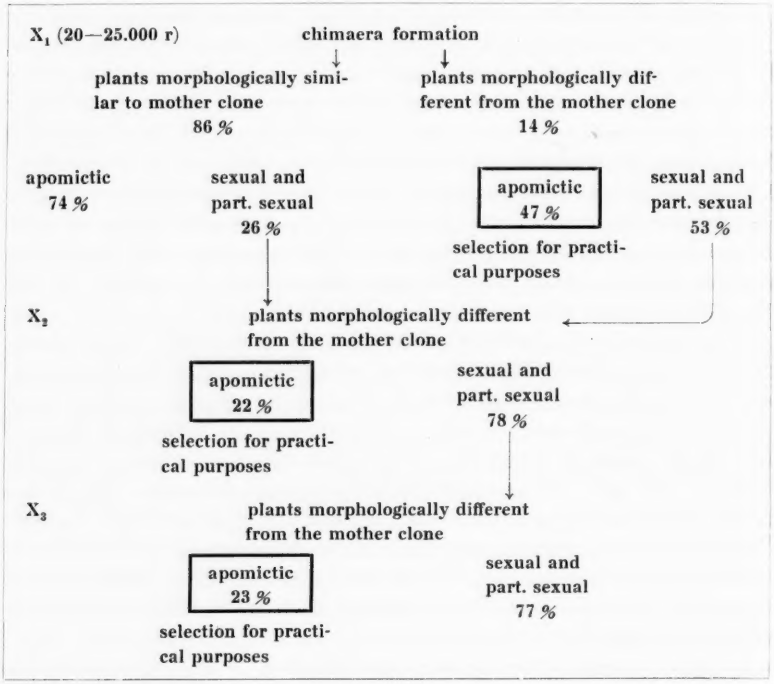
A more pronounced and more easily observed tendency to chimaera formation may perhaps be expected in *Poa pratensis* than in most other species, because of the fact that the species has a great tolerance to different chromosome numbers, and it may thus be expected that plant tissues with large variations in chromosome numbers, and with major chromosome aberrations may survive and compete successfully with unmutated tissues in the plant. Atypical divisions caused by spindle disturbances through irradiation may cause large variations in chromosome number between tissues, giving rise to plant parts different from each other and from the mother plant. Irradiation induced chromosome aberrations might cause loss of one or more chromosomes. Those changes would probably in most cases lead to lethality of the cells concerned in other species, while in *Poa pratensis* also tissues with these changes might be viable in addition to those with induced gene mutations. The possibility of vegetative cloning of the plant makes the observation of the chimaera formation easier than in many species. At the start of this experiment the high probability for chimaera formation was not realised, and vegetative cloning took place only when the plants were fairly old, and weak tissues had perhaps already gone under in competition. It is possible that a larger number of chimaeras had been recovered if vegetative cloning had been made at an earlier stage and single shoots had been taken. In the clonal parts observed the possibility still exists that more than one chimaerical part is present. It is hardly impossible, although the probability is perhaps not very great, that the 12 clonal parts of the X_1-plant investigated embryologically are not quite uniform genetically.

How much in the behaviour of later generations after X-ray treatment is due to the influence of the irradiation cannot be stated. The embryological investigation has shown that the original material used for the treatment must be said to possess a great lability in its seed formation mechanism. As the comparison between the frequency of aberrants in the control material and that in the X-rayed material was made in different years, it can only be said with any certainty that the X-raying has had an influence on the seed formation in those cases where two chimaerical parts of a plant showed different modes of seed formation. The cases of those plants which have been used in the further investigation is fairly low.

However, a scheme of the over all behaviour of plants in successive

generations will be given to show the possibilities for the breeder to select for practical purposes in this material. It can be seen from the scheme, that once variation in the material is brought about through sexual seed formation, apomictic lines may be selected in succeeding generations, where a continuous segregation takes place into sexual and apomictic forms. It must be stressed that the figures given are only applicable to this specifique material, originating from the Fylking clone, and that it cannot be said with any certainty whether the proportion of apomictic to sexual plants in later generations might not be different if only plants where sexuality was with certainty induced by irradiation were included. Some of the X_1-plants whose progeny has been included may belong to the category of aberrant plants normally formed in the progeny of Fylking.

X-irradiation of a facultative apomict with high degree of apomictic seed formation (2 % sexual).



Furthermore, the figures given apply to the progeny after open pollination and as the population of plants grown in the same field has varied from year to year, the genetic background of seed setting in the populations in the different generations is probably quite different depending on the pollen donors.

The possibilities of making controlled crosses between sexual plants or with apomictic father plants also gives the breeder great possibilities. It was shown by the observation of progeny of F_1-plants after crosses of two sexual plants that a fairly large number of apomictic F_1-plants had been produced, some of which also showed good agronomic characters. Starting from an agronomically good clone only a small percentage of the deviating plants produced after X-raying may be expected to be superior to the mother clone and not all of those may be expected to be apomictic. Superior characters may be found already among the chimaeras in the X_1-generation, however, in for instance spreading capacity, length of straw and disease resistance. Plants with better resistance to mildew than the original clone has been found in our material, and HANSSON and JUSKA (1959) produced a stem rust resistant line of *Poa pratensis* by thermal neutron radiation.

SUMMARY

Investigations on the embryology of a clone of *Poa pratensis* have shown the existence of a balanced competition between aposporous and sexual cells during embryosac development, and indicated that the balance may be shifted in favour of one or the other type of cells by environmental agents.

Plant families resulting from X-ray treatment of seeds from the clone have been followed through four generations with embryological and morphological observations. An example of such a plant family is given in Fig. 13. The X_1-plant from the originally irradiated seed was divided vegetatively into nine parts, and was found to be chimaerical. Seed after open pollination was taken from one plant of each of the two chimaerical types, and the progeny (X_2) in both cases found to be variable and deviating from the mother plants. Crosses were made between different father plants and one of the X_1-plants. The progeny was found to be uniform and morphologically identical to the mother plant in one crossing attempt. In two crossing attempts most of the progeny plants were identical to the mother plants, but a few deviated. Renewed seed harvest from the X_1-mother plant after open pollination resulted in pro-

geny that was mainly variable and deviating from the X_1-mother plant. The same X_1-plant had thus behaved as mainly sexual after open pollination in two different years, but as an apomict or partially sexual in crossing attempts.

In advanced generation progeny (X_3 and X_4) after irradiation extreme types both in the direction of sexuality and in the direction of apomixis in embryological behaviour have been recovered, as decided through observation on the embryological development of the plants and observations on the morphology of their progeny. In the extreme sexual types no formation of aposporous embryosac initials has been observed under the experimental conditions, in the apomictic ones the apomictic tendency is manifested both as an increased number of aposporous cells formed per flower, and as an earlier and more often occurring degeneration of the legitimate megaspores. Cases have also been observed where one phase of the apomictic mechanism, the formation of aposporous cells, seems to be working, but not the other, the parthenogenetic development of the cells. In progeny from such plants tetraploid, triploid and diploid aberrant plants have been recovered, but no apomictically formed, morphologically maternal, diploid progeny.

In cases where the embryological observation showed a balanced competition between aposporous and sexual cells, the result of the competition, as decided through morphological observations on the progeny, was sometimes development of the aposporous cells, sometimes of the sexual ones. A survey of the possible ways of seed formation and the places in development where the manner of seed formation may be influenced by different agents is given.

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SELF-INCOMPATIBILITY IN *FESTUCA PRATENSIS* HUDS.

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I. INTRODUCTION

SEVERAL ways are now known in which the barrier between pollination and fertilization in angiosperms is raised by the self-incompatibility genes (BATEMAN, 1952; LEWIS, 1954; PANDEY, 1957). Since the task of establishing the details of an incompatibility system is laborious and time-consuming, the number of well analysed species is low. Nevertheless, uniformity is indicated, closely related species generally showing the same type of system. That this uniformity is fundamental and not a random one is obvious from the unilateral incompatibility between self-incompatible and self-compatible species (LEWIS and CROWE, 1958). The pollen of the self-compatible species may show remnants of a self-incompatibility system, which may lead to incompatibility upon pollination of self-incompatible species, not only from the same, but even from a closely related, family.

Thus, the incompatibility genes seem to be very ancient constituents of the breeding systems in angiosperms (LEWIS and CROWE, 1958). Exceptions to a uniform incompatibility system within taxonomic groups would arouse considerable interest, since they would provide an insight not only into taxonomical affinity and splitting but also into age and evolution of the incompatibility systems themselves.

In this respect the grass family offers a promising hunting ground. The incompatibility system so far observed in grasses — rye and *Phalaris coerulescens*, both diploid species with 2n=14 — differs fundamentally from other angiosperm systems (LUNDQVIST, 1954, 1956; HAYMAN, 1956). The "grass system" involves two multiallelic loci, both with gametophytic action in the pollen, and, in contrast to systems with a one-locus gametophytic determination of the pollen specificities, is unimpaired by polyploidy. The two loci elaborate the incompatibility specificities in a complementary way, but it is highly probable that they have originated by the duplication of a simple one-locus gametophytic

system. In addition to the grasses, at least two cases of a two-loci gametophytic incompatibility system have been established, both clearly indicating a duplicative origin. In *Beta vulgaris* (Chenopodiaceae) both factors of the haploid pollen have to be matched in the pistil in order to effect incompatibility, but there seem to be factorial identities between the loci (OWEN, 1942). In *Physalis ixocarpa* (Solanaceae) a single factor is sufficient for incompatibility, and self-incompatibility is unimpaired in the polyploid (PANDEY, 1957).

In order to see whether the occurrence of two incompatibility loci, as first observed in rye, was only fortuitous, another diploid species with the basic chromosome number=7, but perennial, was deliberately chosen. A preliminary investigation into the incompatibilities in *Festuca pratensis* indicated the presence of a similar system (LUNDQVIST, 1955). The experiments were continued, since a carefully analysed set of perennial tester genotypes permits a more penetrating study of the incompatibility genes and their mode of action than is possible in an annual material.

II. MATERIALS AND METHODS

Festuca pratensis, the meadow fescue, is a diploid perennial with 2n=14 and moderate to strong self-incompatibility (e.g., FRANDSEN, 1917; TROLL, 1931; NILSSON, 1934). Some plants of different provenience in a collection of this species, kindly put at my disposal by Dr. N. O. BOSEMARK, were selfed in 1954, and the I_1 offspring from the two most self-incompatible individuals were selected for further analysis. One of these offspring families, however, had soon to be discarded owing to poor floral development. Consequently, the following incompatibility analysis is based on offspring from one plant only. This plant, 17.Sv.3:95 (BOSEMARK), originates from the "Svalöf late" strain, which is derived from a wild plant found in Västergötland in Southern Sweden (JULÉN and ÅKERBERG, 1951). Incompatibility inter-relationships were assessed from seed-setting and by observation of the pollen behaviour on the pistil.

To study the seed-setting, the plants were cloned; and selfings and intercrosses were done in the field, in double pergamine bags sized 30×10 cm., tightened with cotton in the opening, and attached to a stake. Bagging was done just before anthesis and, as a rule, did not comprise more than two panicles. Seed-setting was gauged in relation to the number of spikelets some three weeks after bagging. However,

TABLE 1. *Seed-set per spikelet upon selfing and sibmating within I_1 54/17.Sv.3:95.*

Upper figure in a square denotes number of bags; placed to the left or right in combinations classed as incompatible or compatible, respectively.

♂ \ ♀	A		B									C	D	E				F		G	H		Mean comp.	inc.
	18	26	4	5	8	9	14	20	23	25	28	10	12	3	16	19	27	6	24	7	2	22		
18	9 .09	3 .15	1 .07	2 .08	5 .24	5 .12	6 .08	4 .09	5 .21	3 .07	4 .05	4 .12	3 .08	3 .06	4 .15	3 .25	4 .07	2 .10	6 .09	4 .14	2 .01	3 .13		.11
26	4 .04	9 .10	4 .10	3 .03	1 .10	2 .06	2 .06	3 .03	1 .07	5 .14	7 .07	3 .06	1 .02	3 .20	1 .09		5 .10	1 .11	2 .13	2 .31	1 .04	2 .03		.09
4	1 .74	4 .61	6 .01	4 .03	4 .08	3 .00	3 .02	4 .03	1 .00	5 .04	2 .05	3 .70	1 .11	7 .64	1 .64	1 .35	2 .95	7 .01	4 .02	7 .05	3 .77	2 1.01	.71	.03
5	2 1.39	3 .40	4 .20	5 .18	1 .27	7 .19	4 .15	3 .37	2 .11	1 .09	4 .22	2 1.39	1 .17	1 .90	1 2.48	2 1.85	2 1.52	5 .17	2 .31	5 .36	1 .61	2 .92	1.27	.22
8	5 .50	1 .59	4 .06	1 .06	7 .15	7 .11	4 .21	4 .23	1 .05	4 .16	5 .06	4 .91	4 .15	3 .94	3 1.54	1 2.97	2 .59	4 .06	1 .02	9 .09	1 .65	2 .80	1.05	.11
9	5 1.35	2 .94	3 .05	7 .20	7 .21	6 .28	4 .05	5 .11	3 .45	3 .25	3 .40	4 1.12	3 .37	2 .65	1 .87	1 1.39	2 1.75	3 .25	5 .08	5 .17	1 .43	5 .57	1.01	.21
14	6 1.00	2 .53	3 .12	4 .03	4 .07	4 .04	6 .02	6 .09	3 .10	6 .06	5 .07	2 1.32	2 .02	1 1.65	3 1.17	1 .61	2 .53	3 .04	6 .05	4 .07		2 .37	.90	.06
20	5 .86	3 .67	4 .05	3 .12	4 .13	5 .16	6 .10	6 .07	5 .10	6 .07	6 .17	4 1.71	3 .18	2 .71	1 2.24	4 .98	2 1.54	4 .04	5 .05	6 .22		3 .77	1.19	.11
23	5 2.33	1 2.76	1 .45	2 .73	2 1.07	3 .74	3 .57	5 .31	5 .32	3 .37	3 .21	2 1.43	5 .40	1 .59	2 1.85	1 1.17	3 1.16	3 .22	6 .55	4 .94		2 .91	1.53	.54
25	3 1.09	5 .35	5 .06	1 .04	3 .13	3 .06	6 .06	6 .15	3 .18	7 .10	7 .08	5 1.27	1 .00		4 1.02	1 .45	3 .42	2 .03	5 .05	5 .14		3 .25	.69	.09
28	4 .80	7 .85	2 .14	5 .04	5 .07	3 .09	5 .08	6 .20	3 .04	6 .02	6 .04	2 1.53	1 .23	3 .82	6 1.54	1 1.20	4 .77	6 .07	3 .08	5 .26		2 .34	.98	.09
10	4 .93	2 .63	3 .42	2 .85	4 .61	4 .78	2 .83	4 .98	2 1.77	5 .89	2 .87	6 .15	3 .10	1 .85	3 .62	1 .73	1 .95	5 .32	2 .58	4 1.09	2 .07	4 .10	.82	.11
12	3 1.35	1 1.33	1 .16	1 1.36	4 1.52	3 1.77	2 1.64	3 2.35	5 1.47	1 1.94	1 1.63	3 1.94	5 .00	4 1.99	2 2.02	1 1.41	1 3.35	3 .01	4 .06	4 2.52	2 .85	1 1.69	1.70	.04
3	3 .56	3 1.67	7 .77	1 1.84	3 .97	2 .88	1 2.51	2 .55	1 1.48		3 1.07	1 .73	4 .26	6 .04	4 .05	6 .10	8 .04	4 .71	1 2.22	3 .03	5 .02	1 .00	1.23	.04
16	4 .94	1 .47	1 1.46	1 1.27	3 .83	1 .63	3 .84	1 2.03	2 1.20	4 1.00	6 .69	3 .55	1 .13	4 .12	5 .08	3 .13	1 .14	1 1.52	1 .83	2 .05	1 .00	1 .06	1.02	.08
19	3 1.45		1 1.40	2 1.34	1 1.79	1 1.44	1 .85	4 1.00	1 1.91	1 1.88	1 1.31	1 1.75	1 .47	6 .17	3 .19	7 .10	1 .09	3 .43	1 .00	2 .00	1 .00	1 .16	1.27	.10
27	4 .93	4 .67	2 .49	2 .62	1 .40	2 1.11	2 .64	2 .69	3 .83	3 .89	5 1.09	1 1.76	1 .06	8 .07	1 .24	1 .16	6 .10	3 .61	5 .92	5 .29	1 .38	6 .18	.83	.20
6	2 1.14	1 .45	7 .48	5 .84	4 .54	3 .79	3 .88	4 .79	3 .99	2 .67	6 1.15	5 .73	3 .28	4 .73	1 .77	3 1.02	3 .89	5 .02	3 .12	4 .81	4 .76	4 .81	.80	.07
24	6 1.04	2 .45	4 .41	2 1.61	1 1.52	5 .45	6 .82	5 .70	6 1.16	5 1.00	3 .87	2 1.04	4 .35	1 1.14	1 1.51	1 .23	5 1.31	3 .06	5 .08	4 1.33	2 1.49	7 .71	.99	.07
7	4 .79	2 .97	7 .46	5 .83	9 .56	5 .69	4 .86	6 1.20	4 1.10	5 1.25	5 1.30	4 1.13	4 .19	3 1.02	2 1.62	2 1.69	5 .88	4 .57	4 .66	6 .06	3 .71	3 .85	.96	.06
2	2 .93	1 2.83	3 .46	1 .12	1 .16	1 .59						2 .05	2 .06	5 .77	1 2.00	1 .43	1 1.40	4 1.29	2 2.15	3 1.45	4 .01	3 .00	1.05	.01
22	3 .87	2 .45	2 .24	2 .46	2 .75	5 .48	2 .48	3 .81	2 .91	3 .35	2 .54	4 1.22	1 .15	1 .64	1 .29	1 .60	6 .54	4 .42	7 .63	3 .64	3 .00	6 .01	.60	.01
	1.05 .07	.93 .13	.61 .12	1.01 .14	.88 .23	.87 .17	1.04 .13	1.11 .15	1.28 .15	1.10 .12	1.05 .13	1.17 .11		.94 .11	1.39 .13	1.07 .15	1.16 .09	.73 .09	1.00 .12	1.31 .20	.78 .06	.77 .07	comp. inc.	

anthesis not being simultaneous among the plants, some contamination by stray pollen during the process of bagging of the latest plants was likely to occur. No emasculations were performed, and the mutual pollination of the paired panicles was only imperfectly controlled by

shaking the bags early in the morning. To select suitable panicles for crosses required great care in order to bring about mutual pollination. Owing to these technical difficulties most of the crosses were repeated up to eight times in 1956—1959. Great care was taken to avoid contamination of the clones by seeds from open flowering.

To check the reliability of this analysis, pollen behaviour on the pistil was studied with the cotton-blue technique (WATKINS, 1925; HAYMAN, 1956). Panicles were brought in from the field and kept in water. A few florets just ready for anthesis were emasculated with forceps, and the other florets removed. For pollination, other panicles were permitted to shed their pollen spontaneously on the widely extruding stigmas of the emasculated florets. It is important that completely fresh pollen be dusted and an excess of pollen on the stigmas avoided, pollen germination being strongly influenced by these factors. The emasculated and thinned panicles may be kept in water for several days without inconvenience. Stigmas were collected 24—36 hours after pollination, with excellent contrast between emptied, compatible, and still almost completely filled, incompatible, pollen grains, and at least 200 pollen grains, where possible, were classified and counted in a pollination.

The statistical calculations are in accordance with BONNIER and TEDIN (1957).

III. EXPERIMENTAL RESULTS

1. Genetic analysis and interpretation

Of the 28 plants in the I_1 family 54/17.Sv.3:95, one was lost and one was probably a contaminant, being self-incompatible but compatible both ways with the parent plant. Three plants were self-compatible and compatible in all cross-combinations, and, for unknown reasons, one self-incompatible plant gave inconsistent data of seed-setting.

The mean values of seed-setting upon selfings and sib intercrosses in 1956—1959 with the remaining 22 plants are entered in Table 1. Self seed-set ranges from 0 to 0.32 seeds per spikelet, the majority of the values being close to 0.10. (The number of florets in a spikelet may vary from 4—12, the mean being about 6.) Seed-set from intercrosses ranges from 0 to 3.35. From the pattern of cross seed-set, eight groups, *A*—*H*, may be distinguished, numbering 1—9 plants, which are incompatible within, and compatible at least in one direction between groups. Both as male and female the plants average with a clear difference in

TABLE 2. *Seed-set summarized from Table 1.*

Presumably compatible and incompatible crosses separated and compared with open seed-set.

Pollination	Number of seeds per spikelet														n
	0.00	0.25	0.50	0.75	1.00	1.25	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25	3.50	
Self	19	2														21
Cross, incomp.	148	16	4	1	1											170
„ , comp.	7	30	53	63	29	28	18	14	6	3	2	3	—		1	257
Open flowering	2	—	1	4	3	4	2	3	1	—	2					22

seed-set between compatibility and incompatibility. Plant 12, however, was a poor pollen producer and unreliable as male.

Within the 257 crosses presumed to be compatible (pollinations by plant 12 being excluded), there is a wide range of variation. In fact, the overlapping between compatible and incompatible crosses is rather pronounced (Table 2). Although open seed-set for the 22 plants shows a similar overlapping (data from 1959, three panicles/plant), it may be asked whether these inconsistencies of the classification in Table 1 are real, which would indicate that an intra-incompatible group may consist of more than one genotype.

The classification may be checked by an analysis of the behaviour of the pollen in doubtful crosses. Preliminary observations showed that in *Festuca pratensis* incompatibility leads to an early inhibition of pollen tube growth. Thus, a genetically incompatible pollination would lead to no, or almost no, emptied pollen. Ranging from 0.25 to 1.07

TABLE 3. *Frequency of emptied pollen grains in cross combinations where seed-set was not unambiguously consistent with the incompatibility grouping in Tables 1 and 2.*

(Doubtfully incompatible=0.25—1.25. Doubtfully compatible=0—0.50.)

Previous classification	Pollen plant	Per cent emptied pollen grains										n
		0	10	20	30	40	50	60	70	80	90	100	
Incompatible	Original	12	1										13
„	Substituted	9											9
Compatible	Original			1	1	3	4	2	1	1	1		14
„	Substituted		1	2	5	3	6	3	1	1	1		23

seeds/spikelet, 22 presumably incompatible crosses were compared with 37 crosses classed as compatible but only ranging from 0 to 0.50 seeds/spikelet (Table 3). In the latter category a clearly higher proportion of emptied pollen grains was found. In almost all incompatible crosses, some emptied pollen grains could, however, be seen. Contamination by stray pollen is not unlikely, but it sometimes seems that the rigour of incompatibility is reduced at the distal part of the stigma: in any case the unexpected emptied pollen grains are concentrated to this region. In about half of the tests, the original pollinator was substituted by some other plant which, however, had probably the same genotype. We can therefore conclude that the classification in Table 1 is not likely to lead to real inconsistencies.

Thus, each of the eight groups *A*—*H* seems to correspond to a single incompatibility genotype, and more than one locus controlling the incompatibility specificities must be postulated. The pattern of one-way incompatibilities divides the eight groups into three categories: *A* is incompatible as female with all other groups, and *B*—*E* constitute the female incompatible component in combinations with *F*—*H*. Postulating two incompatibility loci, these three categories nicely correspond to the three possible degrees of heterozygosity in an I_1 family, *A* being heterozygous at both loci, *B*—*E* heterozygous at either of the loci, and *F*—*H* homozygous at both loci.

The parent plant being incompatible as female with its I_1 offspring (not all offspring were tested, however), sporophytic control of the pistil specificities with independent action of the alleles is indicated. Considering the control of the pollen specificities, the data are compatible with three interpretations (for a detailed discussion, cf. LUNDQVIST, 1956, pp. 301—309). They require for incompatibility that all those factors are matched in the pistil that — either autonomously (gametophytically) or maternally (sporophytically) — determined the incompatibility phenotype of the particular haploid pollen grain. (The notation refers to LUNDQVIST, 1956.)

Ia. Gametophytic control at both loci. Two determinants, one at each locus and both carried by the pollen.

IIa. Sporophytic control at both loci with independent action of alleles. At least two and maximally four determinants of the pollen.

IVa. Sporophytic control at one locus with independent action of alleles, gametophytic control at the other locus. At least two, and maximally three, determinants of the pollen.

These schemes lead to identical patterns of incompatibility inter-

TABLE 4. *Average seed-set and genetic interpretation of Table 1.*

♀ \ ♂	A	B	C	D	E	F	G	H	Inc. genotype S	Z
A	◇ .10	◇ .09	◇ .09		◇ .13	◇ .11	◇ .23	◇ .05	1.2	3.4
B	c .99	◇ .16	a 1.26	c	c 1.16	◇ .12	◇ .26	a .65	1.1	3.4
C	c .78	a .89	◇ .15	c	c .79	a .45	a 1.09	◇ .09	2.2	3.4
D	d 1.34	d 1.54	d 1.94	◇ .00	a 2.19	◇ .04	a 2.52	a 1.27	1.2	3.3
E	d .96	d 1.13	d 1.20	a	◇ .11	a .91	◇ .09	◇ .10	1.2	4.4
F	b .77	d .87	d .89	c .32	c .95	◇ .07	a 1.07	a .94	1.1	3.3
G	b .88	d .92	d 1.13	c	c 1.30	a .62	◇ .06	a .78	1.1	4.4
H	b 1.27	d .49	d .64	c	c .83	a 1.12	a 1.05	◇ .01	2.2	4.4

◇ = incompatibility
⊡ = compatibility
□ = data unreliable

a – d = type of compatible pollination

relationships with 60.9 % compatible sib intercrosses in an I_1 family. Disregarding the pollinations by plant 12, the number of compatible sib intercrosses in the present material corresponds to 60.2 %.

Denoting the two loci as *S* and *Z* respectively, as done in previous cases (LAWRENCE, 1930; OWEN, 1942; LUNDQVIST, 1954; HAYMAN, 1956; PANDEY, 1957), and designating the selfed parent plant as $S_{1.2}Z_{3.4}$, the genotypes corresponding to the pattern of incompatibilities have been entered in Table 4.

The pollen itself will indicate the way in which its incompatibility specificities are genetically determined. At sporophytic determination, the individual pollen genotype does not matter, all pollen grains from a given plant being maternally determined with the same incompatibility phenotype and displaying a uniform behaviour. At gametophytic determination, the individual pollen genotype determines the phenotype and effects an individual behaviour of the pollen grains. When

TABLE 5. *Proportion of emptied pollen grains observed upon sibmating within I_1 family 54/17.Sv.3:95, in relation to the percentage of compatible pollen expected when two incompatibility loci both exert the control of the pollen gametophytically, sporophytically, or one locus sporophytically, the other gametophytically (types Ia, IIa, and IVa in* LUNDQVIST, *1956).*

The tabulation assumes that *S* is the sporophytic locus in IVa.

Pollination type in Table 4	Observed per cent emptied (compatible) pollen grains																	
	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
a							1	1	—	8	5	7	4	7	8	6	4	1
b							1	1	2	5	—	2	2	—	—	—	1	
c				2	5	8	12	8	10	9	1							
d		1	1	—	4	9	9	9	7	3	1							

v^2=83.742; df 3/161; P<0.001.

Pollination type	Per cent compatible pollen					Number of pollinations
	expected on			observed		
	Ia	IIa	IVa	$\bar{x}$	±m	
a	100	100	100	68.48	2.196	52
b	75	100	100	56.07	8.157	14
c	50	100	100	40.68	2.076	55
d	50	100	50	38.64	2.595	44

Statistical analysis		
Difference	t	P
a—b	3.851	<0.001
c—d	0.946	0.4—0.3

one locus has sporophytic, the other gametophytic, determination of the pollen, there may be individual behaviour of the pollen or the parallel between genotype and phenotype of the pollen may be broken, all depending on the genotypic conditions at the particular cross (LUNDQVIST, 1956, p. 327).

Without going into the genotypic details (which can be derived from Table 4), compatible pollinations within an I_1 family, segregating at both incompatibility loci, can be divided into three categories. All pollen will be compatible on

(1) any one of the three hypotheses (pollination type *a* in Table 4),

(2) hypotheses IIa and IVa (pollination types *b* and *c*),

(3) hypothesis IIa, only (pollination type *d*). (By hypothesis IVa it is assumed that *S* is the sporophytic locus. With *Z* as the sporophytic locus, pollination types *c* and *d* interchange their places in Table 4, but this does not influence the following discussion.)

Pollen behaviour was studied in 165 compatible cross combinations in which all four pollination types *a*—*d* were represented (Table 5). Although the proportion of emptied pollen grains had a wide range of variation, there were clear differences between the pollination types. Agreement with expectation was only obtained for hypothesis Ia, which assumed gametophytic determination of the pollen at both loci. Pollination type *b*, compared to *a*, was expected to lead to a lower proportion of compatible pollen only on this hypothesis; and *a* yielded significantly more emptied pollen grains ($t=3.851$; $P<0.001$). Pollination type *d*, compared to *c*, was expected to lead to a reduced proportion of compatible pollen grains on hypothesis IVa: the difference is not significant.

The proportions of emptied pollen grains are almost constantly below the values expected on the genotypic scheme in Table 4, the expected values of 50, 75, and 100 % compatible pollen grains corresponding to the observed mean values of about 40, 56, and 68 % emptied pollen grains, respectively. However, the general trend of the data reasonably agrees with what is expected when determination of the pollen is purely gametophytic. Considering the many environmental and physiological factors that, in addition to the incompatibility interaction, may negatively influence the growth of the pollen, the deficit of emptied pollen grains is not surprising.

In an I_1 family segregating at two loci, the expected frequencies of the nine possible genotypes will be as follows: $^1/_4$ of the plants are heterozygous at both loci (het het); four genotypes, each with $^1/_8$ of the plants, are heterozygous at only one locus (ho het); four genotypes, each with $^1/_{16}$ of the plants, are homozygous at both loci (ho ho). In Table 1, by the criterion of seed-setting, a classification of 22 plants was made. Four other plants were classified by the criterion of pollen behaviour with the following result: plant 11, self-incompatible, genotype $S_{1.2}Z_{3.4}$; plants 15, 17, and 21, all self-compatible with loss of the activity in the pollen, genotypes $S_{1.2}Z_{3.4}$, $S_{1.2}Z_{4.4}$, and $S_{1.1}Z_{3.4}$, respectively.

The fact that 10 out of 26 plants were presumed to belong to the same ho het genotype $S_{1.1}Z_{3.4}$ deserves some consideration. The number of plants involved in the analysis is too small to permit a decision as to whether the deviation from expectation is due to more than chance alone, but since it might well be a case of hidden heterogeneity that was not possible to detect with the aid of seed-setting data, group *B* of Tables 1 and 4 was checked by observations of pollen behaviour on the pistil.

The four ho ho genotypes form a very efficient tester set. In the

TABLE 6. *Frequency in per cent of emptied pollen grains in test pollination of the presumed group $S_{1.1}Z_{3.4}$ in I_1 54/17.Sv.3:95 (group B in Tables 1 and 4).*

Pollen plant			Pollinated plant no.									
S	Z	no.	4	5	8	9	14	20	21	23	25	28
1.1	3.3	24	5.7	8.2	2.2	5.1	8.2	8.5	2.4	1.2	3.4	1.8
1.1	4.4	7	2.5	4.6	2.2	1.8	11.0	4.1	2.2	5.0	3.1	5,3
2.2	3.4	10	73.6	88.9	78.9	82.7	76.6	79.4	83.3	88.7	72.1	78.2
2.2	4.4	22	59.2		57.1	38.0	50.5	64.8	52.9		51.5	52.1

segregating I_1 family, the het het genotype leads to complete inhibition of the pollen of all four testers, a ho het genotype is incompatible with two of them, and a ho ho genotype is compatible with all but its own genotype among the testers. The direct identification of all nine genotypes which can be formed in the I_1 family is, thus, possible.

Three of these testers were available: no. 24 = $S_{1.1}Z_{3.3}$, no. 7 = $S_{1.1}Z_{4.4}$, and no. 22 = $S_{2.2}Z_{4.4}$. The genotype $S_{2.2}Z_{3.3}$ was substituted by $S_{2.2}Z_{3.4}$ = no. 10. A plant with the genotype $S_{1.1}Z_{3.4}$, upon pollination by this tester set, will react incompatibly with the first two, and be compatible with the latter two testers. The pattern of reaction to the tester set did not indicate any heterogeneity within group *B* (Table 6). Tester no. 22 had an unexpectedly low proportion of emptied pollen grains, due possibly to its poor vitality.

2. Segregation disturbances of the incompatibility genes

The frequency with which a particular incompatibility gene is recovered upon selfing is primarily due to the rigour of the incompatibility interaction which it controls. Secondarily, homozygosity for deleterious genes may lead to elimination of particular incompatibility genotypes. Such deleterious genes tend to accumulate close to the incompatibility locus when homozygosity for the incompatibility genes is prevented.

In the present two-loci system where there is no bar to homozygosity for incompatibility genes, the frequency with which these genes are recovered upon selfing most likely indicates the ease with which the incompatibility barrier is overcome. Does this ease depend on the individual *S* or *Z* gene or on the particular *S*—*Z* pair? It is obvious that a study of disturbed segregation ratios of the incompatibility genes may

TABLE 7. *Genotypic analysis in I_1 59/17.Sv.3:95, by the criterion of pollen behaviour on the pistil when four tester plants pollinate.*

+=many emptied pollen grains (100 % expected).
—=few or no emptied pollen grains (0 % expected).

	Locus		Genotypes of pollinated plants								
	S	Z	1.2 3.4	1.1 3.4	2.2 3.4	1.2 3.3	1.2 4.4	1.1 3.3	1.1 4.4	2.2 3.3	2.2 4.4
Genotypes of pollen plants	1.1	3.3	—	—	+	—	+	—	+	+	+
	1.1	4.4	—	—	+	+	—	+	—	+	+
	2.2	3.4	—	+	—	+	+	+	+	+	+
	2.2	4.4	—	+	—	+	—	+	+	+	—
Total in 1959			16	3	5	4	8	—	4	—	5
„ „ 1954			4	10	1	1	5	2	1	—	2

yield important information on the activity of the genes in this two-loci system.

In order to see whether the excess of ho het $S_{1.1}Z_{3.4}$ was due to chance alone, 48 plants were investigated in a second I_1 family derived in 1959 from plant 17.Sv.3:95. In 1960 these plants were classified by the criterion of pollen behaviour on the pistil, according to the method outlined above. In the tester set, however, no. 22 was substituted by no. 2 ($=S_{2.2}Z_{4.4}$), and no. 10, flowering too late to be used in the tester set, was substituted by a plant derived from the cross no. 22 × no. 10 ($=S_{2.2}Z_{4.4}\times S_{2.2}Z_{3.4}$) and checked to have the genotype $S_{2.2}Z_{3.4}$. As may be seen from Table 7, the genotypes $S_{1.2}Z_{3.3}$ and $S_{1.1}Z_{3.3}$ react alike upon pollination by this tester set. $S_{1.2}Z_{3.3}$, however, in contrast to $S_{1.1}Z_{3.3}$, inhibits half of the pollen of $S_{2.2}Z_{3.4}$ and successfully pollinates $S_{1.1}Z_{3.3}$.

The conditions of pollination seemed to be less favourable in 1960 (the expectation of 100 % compatible pollen grains corresponding to an average of about 60 % emptied grains), and there was a wide range of variation within the category of compatible pollinations. In only 2 % of the cases, however, was it necessary to repeat the pollination, and the conclusions could be based on a completely consistent pattern of reactions. Owing to poor vegetative development, three of the yearling plants were left undetermined. The other 45 plants are entered in Table 7 as are also, for comparison, the 26 plants of 1954.

In the new I_1 family there is no excess of ho het $S_{1.1}Z_{3.4}$, and het het $S_{1.2}Z_{3.4}$ is the largest group. The absence of ho ho $S_{2.2}Z_{3.3}$ in both families

TABLE 8. *Frequencies of the three degrees of heterozygosity within I_1 17.Sv.3:95. Expected distribution 1:2:1.*

Material	het het	ho het	ho ho	Total	χ^2(1:2:1)	P
1954	4	17	5	26	2.539	0.3—0.2
1959	16	20	9	45	2.734	0.3—0.2
Total	20	37	14	71	1.140	0.7—0.5

$\chi^2_{het(1:2:1)}=4.133$; $0.2>P>0.1$.
$\chi^2_{het}=3.773$; $0.2>P>0.1$.

is interesting. The materials are, however, too meagre for a direct comparison of their distribution of genotypes, and some kind of pooling is required.

With undisturbed segregation the three genotypic categories het het, ho het, and ho ho are expected to occur in the proportions 1:2:1 among the I_1 plants; disturbances of these proportions would reflect disturbed frequencies of the pooled individual genotypes. Neither of the I_1 families was found to deviate significantly from expectation, nor could they be demonstrated to differ more from one another than might be due to chance alone (Table 8). Here it is of particular interest to note that there is no indication of a deficit in the ho het category, since this demonstrates the independent inheritance of the *S* and *Z* genes. With complete linkage between these two loci, het het and ho ho would be the only categories in the I_1 family.

The absence of significant disturbances at the level of heterozygosity does not exclude disturbances at the level of the individual zygotic genotypes. In the pooled material with 71 I_1 plants, the deviations from the expected genotypic frequencies might well be due to chance alone (Table 9). In view of the rather poor agreement at the level of heterozygosity between the I_1 families, this pooling might be unwarranted,

TABLE 9. *Distribution of the nine incompatibility genotypes within pooled I_1 17.Sv.3:95.*

Incomp. S / genotype Z	1.2 3.4	1.1 3.4	2.2 3.4	1.2 3.3	1.2 4.4	1.1 3.3	1.1 4.4	2.2 3.3	2.2 4.4	Total
Obs. frequ.	20	13	6	5	13	2	5	—	7	71
Exp. prop.	4	2	2	2	2	1	1	1	1	16

$\chi^2=14.069$; $0.10>P>0.05$.

TABLE 10. *Frequency with which the four S—Z gene pairs are recovered upon pseudo-compatibility of 17.Sv.3:95.*

I_1 plants with the genotype $S_{1.2}Z_{3.4}$ have to be excluded from this analysis. Expected distribution 1:1:1:1.

Material	Gene pair				Total	χ^2(1:1:1:1)	P
	$S_1 Z_3$	$S_1 Z_4$	$S_2 Z_3$	$S_2 Z_4$			
1954	15	17	2	10	44	11.946**	0.01—0.001
1959	7	19	9	23	58	12.345**	0.01—0.001
Total	22	36	11	33	102		

χ^2_{het}=10.880*; 0.02>P>0.01.

however, and real disturbances of the genotypic frequencies would seem to be probable.

The frequency with which the four gametic *S—Z* gene pairs are recovered from the selfed parent plant constitutes the next level at which disturbed segregation ratios may be looked for within the I_1 progeny. This analysis is possible in ho ho and ho het individuals, since in these cases only one pair of gametic genotypes is able to lead to the particular zygotic genotype (e.g., $S_{1.1}Z_{3.3}=S_1Z_3+S_1Z_3$; $S_{1.1}Z_{3.4}=S_1Z_3+S_1Z_4$). Het het plants can be formed in two ways ($S_{1.2}Z_{3.4}=S_1Z_3+S_2Z_4$ or $S_1Z_4+S_2Z_3$) and have, thus, to be excluded from the analysis.

In the material selected in this way, with undisturbed segregation the four *S—Z* gene pairs are expected to be equally common. Both families show highly disturbed ratios of gene pairs, but the two patterns of ratios are not similar, S_1Z_3 being in excess in 1954 and in minority in 1959, S_2Z_4 showing inverse ratios, and S_1Z_4 and S_2Z_3 being both years in excess and in minority, respectively (Table 10).

These disturbed ratios at the level of the *S—Z* gene pair do not necessarily imply that the rigour of the incompatibility interaction between pollen and pistil depends rather on the particular pair of *S—Z* genes in the pollen grain than on the individual *S* and *Z* genes themselves. Disturbed ratios of the *S—Z* gene pairs might follow in both cases. When due to the action of *S—Z* gene pairs, such disturbances may or may not be accompanied by disturbed proportions among the recovered genes. On the other hand, when the efficiency with which a pollen grain is incompatibly inhibited depends on the presence of a particular *S* or *Z* gene, there will then be disturbed segregation ratios both of genes and of gene pairs. Thus, if a disturbed ratio is only found at the level of the

TABLE 11. *Frequencies of the four individual incompatibility genes within I_1 17.Sv.3:95.*

Factor *a* denotes S_1 or Z_3, *b* denotes S_2 or Z_4. Expected distribution of alleles 1:1.

	Material	Incompat. factor a	Incompat. factor b	Total	χ^2(1:1)	P
S-locus	1954	36	16	52	7.692**	0.01—0.001
	1959	42	48	90	0.400	0.7—0.5
	Total	78	64	142		

χ^2_{het}=6.779**; 0.01>P>0.001.

Z-locus	1954	21	31	52	1.924	0.2—0.1
	1959	32	58	90	7.512**	0.01—0.001
	Total	53	89	142	9.126**	0.01—0.001

χ^2_{het}=0.329; 0.7>P>0.5.

S—Z gene pairs, this indicates that the particular *S—Z* pair is what matters in the incompatibility interaction between pollen and pistil.

With undisturbed segregation, the four incompatibility genes S_1, S_2, Z_3, and Z_4 are expected to be equally common in the I_1 progeny. In the present two I_1 families, disturbed ratios upon selfing were found, Z_3 being in minority in both years, and S_2 being in minority in 1954 (Table 11). It is interesting that in 1959 the segregation at *S* was completely different, S_2 now occurring in a slight (and insignificant) excess.

3. Genetic control of pseudo-compatibility

Pseudo-compatibility is the formation of seed after pollination with genetically incompatible pollen. From the fact that they were not equally frequently recovered upon pseudo-compatibility (Tables 10 and 11) it must be concluded that the *S* and *Z* genes influence in some way the readiness with which the incompatibility barrier is overcome. Pseudo-compatibility, however, might also be controlled by other genes which influence the interaction between pollen and pistil or the female chain of reactions involved in seed formation. The genotypic influence on pseudo-compatibility has been studied in more detail in Table 12.

Excluding the pollinations by no. 12, but including selfings, there are 191 combinations classed as incompatible in I_1 54/17.Sv.3:95 (see Table

TABLE 12. *Seed-set (seed per spikelet) of incompatible matings (sibbings and selfings) within I_1 54/17.Sv.3:95.*

(For individual data, see Table 1.)

Incomp. genotype S / Z	1.2 3.4	1.1 3.4	2.2 3.4	1.2 3.3	1.2 4.4	1.1 3.3	1.1 4.4	2.2 4.4
Mean, ♀ side	0.10	0.16	0.11	0.04	0.11	0.07	0.06	0.01
Mean, ♂ side	0.10	0.15	0.11	—	0.12	0.10	0.20	0.07

Analysis of variance:

Source of variation	I. Female side d. f.	Sum of squares	Mean square	II. Male side d. f.	Sum of squares	Mean square
a. Between genotypes	7	0.2514	0.0359	6	0.2099	0.0350
b. Within genotypes	183	4.0291	0.0220	184	4.0706	0.0221
c. Between plants	14	2.4065	0.1719	14	0.1263	0.0090
d. Within plants	169	1.6226	0.0096	170	3.9443	0.0232
Total	190	4.2805		190	4.2805	

I. Variance ratios	P	II. Variance ratios	P
c/d=17.906***	<0.001	d/c=2.5778*	0.05—0.01
c/a= 4.788*	0.05—0.01	a/b=1.5837	0.2—0.05

1). Means of seed-set in incompatible combinations when the eight genotypic groups obtained served as male and as female, respectively, are given in Table 12. On the female side these means range from 0.01 to 0.16, and on the male side from 0.07 to 0.20.

An analysis of variance has been carried out according to the scheme in Table 12. The male and the female components are studied separately in the 191 incompatible combinations; and eight genotypic groups, numbering 1—9 plants, are represented.

No influence whatever of the male plant on pseudo-compatibility can be observed. On the other hand, the choice of the female plant is of great importance, but this strong influence on pseudo-compatibility cannot be related to a specific incompatibility genotype.

Thus, pseudo-compatibility is predominantly controlled by genotypic conditions in the pistil, and by genes other than *S* and *Z*. The minor influence of the *S*—*Z* genes on pseudo-compatibility is indicated also by the correlation between pseudo-compatibility and general fertility. The means of seed-set at compatible and incompatible combinations are positively correlated when the 22 plants serve as female, $r = +0.4124$; the correlation between seed-set upon open flowering and compatible bagging corresponds to $r = +0.4032$. Neither of these coefficients reach the level of statistical significance ($0.1 > P > 0.05$ in both cases), but a true correlation seems probable.

IV. DISCUSSION

Within subfamily Festucoideae of Gramineae the three tribes Festuceae, Hordeae, and Phalarideae (PRAT, 1936) have displayed a two-loci system for the control of self-incompatibility. In view of its complexity this system is not likely to have arisen more than once in the history of Gramineae and would appear to be of ancient origin, present at least in the beginning of the subfamily Festucoideae. It seems highly desirable to investigate self-incompatibilities of the other subfamilies of Gramineae. Preliminary data from Commelinaceae, which is considered closely related to the Gramineae, indicate the presence of a one-locus gametophytic incompatibility system in *Tradescantia paludosa* (LUNDQVIST, unpublished).

The present data in *Festuca pratensis* clearly demonstrate that the incompatibility specificities in the pollen are determined by factors carried in the pollen grain itself. The pollen in a particular pollination does not always react in a uniform way, and the pattern of failure and success of the pollen as observed cytologically shows a close parallel with the pattern of pollen genotypes as postulated from an independent analysis by the criterion of seed-setting. Even in incompatible pollinations data speak against a uniform behaviour of the pollen such as expected with sporophytic determination of its phenotype, since the incompatibility genes show disturbed segregations upon pseudo-compatibility.

In all three grass species so far investigated there is direct evidence that the *S* and *Z* factors are both active at the gametophyte stage and not in the way that one locus is active sporophytically, the other locus gametophytically. It is important to note that the incompatibility inhibition of pollen as observed cytologically in *Phalaris coerulescens*

(HAYMAN, 1956) and the present material of *Festuca pratensis* leads to genetical consequences of exactly the kind observed in *Secale cereale* (LUNDQVIST, 1956 p. 327, 1958 p. 243).

In all three species the incompatibility interaction requires that both incompatibility factors of the haploid pollen be matched in the pistil, and there is, thus, some functional dependence between *S* and *Z*. Only in rye, by means of the incompatibilities in diploid pollen, could something of the nature of this functional dependence be revealed: *S* and *Z* in some way elaborate a common incompatibility product, and when more than one specific *S* and/or *Z* allele are present, each specific *S*—*Z* pair of genes leads to one specific product. Identity between pollen and pistil in one such pair of genes leads to incompatibility (LUNDQVIST, 1957). The details of this complementary co-operation between *S* and *Z* are unknown.

To explore this question in the present material is of course a hazardous task, since we do not yet know whether the complementary co-operation between *S* and *Z* is present here, too. If, as seems quite probable (LUNDQVIST, 1954, 1958) the two-loci gametophytic system arose as a duplication of a one-locus gametophytic system, the complementary interaction between the two incompatibility loci was not likely to become successfully established at once, and more than one evolutionary pathway might have been followed. For the purpose of the following discussion, however, it will be assumed that in *Festuca pratensis*, also, *S* and *Z* interact in a complementary way.

The definite incompatibility specificity depends on an interaction between specific *S* and *Z* genes. The less the properties of this specificity can be demonstrated to depend on the individual *S* and *Z* genes, the more radical the complementary interaction between *S* and *Z* would appear to be. The rigour of the incompatibility reaction is one such most apparent property and will be reflected by the frequency with which the particular controlling gene or gene combination are recovered in the offspring upon pseudo-compatibility.

When of the four gametic *S*—*Z* gene pairs two with no factors in common (e.g., S_2Z_3 and S_1Z_4) are recovered in minority (or excess) in the self offspring, it would appear that the particular *S*—*Z* pair is what matters. In the present case, S_1Z_4 being in excess and S_2Z_3 in minority (Table 10), there is no safe judgment. To decide whether the different rigour of the incompatibility reaction is due to the individual factor *per se* or to the particular *S*—*Z* pair is a matter of probabilities, and the *S* and *Z* genes should first be studied in several other combinations.

Both *S* and *Z* show disturbed segregations. This might indicate that in relation to the other neither locus exerts a predominant influence on the formation of the incompatibility specificity, which in its turn could be taken to indicate a radical interaction between the loci. However, *Z* in contrast to *S* shows a consistent disturbance of its segregation pattern in I_1 of 1954 and 1959. Z_3 was in minority in I_2 offspring also, obtained from $S_{1.1}Z_{3.4}$, 35 plants in six I_2 families segregating as follows: 21 plants $S_{1.1}Z_{3.4}$, 3 plants $S_{1.1}Z_{3.3}$, and 11 plants $S_{1.1}Z_{4.4}$ ($\chi^2_{(2:1:1)}=5.058$; $0.1>P>0.05$). To draw conclusions as to the relative importance of the two loci *S* and *Z* in the determination of the incompatibility specificities seems highly speculative, in view of the very meagre material and the strong influence found to be exerted on the pseudo-compatibility by genes at other loci.

The question whether there might be any correlation between the degree of pseudo-compatibility and the appearance of the pollen on the pistil needs more critically controlled pollination conditions. The risk of stray pollen could not be eliminated, and, judging from the rather imperfect germination of compatible pollen, the physiological conditions were not quite satisfactory. Emptied pollen grains, with a mean of about 5 %, could be seen in almost all pollinations expected to be incompatible. As was the case in rye (SEARS, 1937) but not in *Phalaris coerulescens* (HAYMAN, 1956), these emptied grains were concentrated to the distal part of the pistil; incompatible tubes were clearly able to grow a considerable way in the stigmatic branches. However, plant 23 which had the highest degree of seed-set upon pseudo-compatibility, was not at all exceptional in frequency and degree of pollen germination, neither when pollinating, nor when being pollinated in incompatible combinations.

In neither of the three investigated grass species is there any evidence of close linkage between *S* and *Z*; and in Secale and Festuca in any case, these loci seem to be independently inherited. The material is meagre but in accordance with expectation. The two-loci gametophytic system with complementary interaction between *S* and *Z*, when compared to the one-locus gametophytic system, shows an increase of general cross-compatibility and a better protection of incompatibility factors against loss by random drift (LUNDQVIST, 1954). This selective advantage of the two-loci system would be lost parallel to the degree that *S* and *Z* were linked, and complete linkage would make the two systems identical.

The "grass system" shows traits which are characteristic of a sporo-

phytic system: (1) Persistence of incompatibilities at the polyploid level (LUNDQVIST, 1954, 1957). In contrast to the sporophytic system the gametophytic incompatibility system is often broken down by polyploidy, and it would appear that selection has had no opportunity in the haploid pollen to balance the interaction between "gametophytic" incompatibility genes (LEWIS, 1943, 1947; ATWOOD and BREWBAKER, 1953). (2) The mature pollen is trinucleate. At anthesis, sporophytically controlled pollen in homomorhphic species is trinucleate in contrast to "gametophytic" pollen which has only two nuclei (BREWBAKER, 1957), and it would appear that the sporophytic activity of the incompatibility gene and the trinucleate state of the pollen both reflect some kind of precocity (PANDEY, 1958, 1960).

The incompatibility system observed in the three investigated grass species is clearly aberrant. The pollen is trinucleate but the incompatibility system is gametophytic, and in spite of the gametophytic control of the pollen, the system is not broken down by polyploidy. Instead of one incompatibility locus, there are two.

It has been suggested that this system has arisen by means of a duplication of a one-locus gametophytic system, whereby in the haploid pollen already there would be an opportunity of balancing the interaction between incompatibility alleles. This, however, does not explain the trinucleate state of the pollen. The aberrant relationship between cytology and incompatibility determination of the pollen has been interpreted in such a way as to suggest that the gametophytic control of the pollen might be the only possible one, if the successive cell wall formation occurring in grasses at microsporogenesis inhibits the free distribution of incompatibility gene products in the cytoplasm of the PMC (PANDEY, 1958, 1960). If the incompatibility genes in grasses originally acted sporophytically, this could also explain that the "grass system" is not broken down by polyploidy. Experiments are being carried on in order to throw light on the origin and mode of operation of this incompatibility system.

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SUMMARY

The genetic control of self- and cross-incompatibilities in *Festuca pratensis*, a diploid perennial grass with 2n=14, is exerted by two probably independently inherited and multiallelic incompatibility loci, as

shown by incompatibility inter-relationships between 71 I_1 offspring derived upon pseudo-compatibility from a single plant of the strain "Svalöf late". Both loci, *S* and *Z*, determine the pollen specificities gametophytically, as assessed from the pattern of failure and success in seed-setting upon sib intercrosses, and by cytological observation of the pollen behaviour on the pistil. Incompatibility requires that both incompatibility factors of the pollen are matched in the pistil, but the nature of this functional dependence between *S* and *Z* could not be established in the present material. As has been demonstrated for a similar case in rye, it might be assumed that *S* and *Z* in some way elaborate a common incompatibility product. Both *S* and *Z* show disturbed segregations upon pseudo-compatibility. This might indicate that compared to one another neither locus exerts a predominant influence on the formation of the incompatibility specificity which in its turn could be taken to indicate a radical interaction between the loci. But, on the other hand, the pattern of segregational disturbances is not consistent in all respects with this suggestion. It also turns out that pseudo-compatibility is predominantly controlled by genotypic conditions in the pistil and only to a minor degree by the *S* and *Z* genes themselves. Other facts relating to the origin and mode of operation of this two-loci system are discussed.

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CENTROMERE-LIKE BEHAVIOUR OF NON-CENTROMERIC BODIES

I. NEO-CENTRIC ACTIVITY IN CHROMOSOME ARMS AT MITOSIS

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INTRODUCTION

MITOSIS is a complicated process, and the movements and behaviour of the chromosomes during its course are determined by many different factors that work more or less independently of one another. The main factor that is responsible for the regular distribution of the chromosomes to the daughter nuclei consists in the pulling action of the chromosomal spindle fibres. In normal chromosomes of most organisms these fibres extend from a small specialized region called the centromere or kinetochore. Chromosomes of this kind are called monocentric. In a few special organisms chromosomes of another type are found, in which chromosomal spindle fibres extend from the chromosomes along their whole length. Such chromosomes are said to be holokinetic or to have a diffuse kinetochore (diffuse centromere). Another organization type again may be represented by *Ascaris megalocephala* in which the middle region of each chromosome is thought to have a number of localized centromeres, *i.e.* the chromosomes are multicentric. It is an open question, however, whether there is a real difference between the holokinetic and the multicentric types of organization, because it is quite possible that the chromosomes also in the holokinetic type may not have a completely continuous centromere along their whole length but rather a series of numerous small localized regions of activity.

There are a number of observations on ordinary monocentric chro-

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mosomes showing that also other regions of these chromosomes besides their centromeres are able to move actively on the spindle in a way similar to that of the centromeres (ÖSTERGREN, 1962). This centromere-like activity of other chromosome regions was called *neo-centric activity* by RHOADES (1952) who also called the regions showing this behaviour neo-centromeres. In the present paper the term neo-centric activity will be used, but we are not going to use the term neo-centromere. We feel that this might give the reader the impression that we are concerned with a new-formation of centromeres or that the neo-centromeres might be especially differentiated and developed organs of the chromosomes such as is the case with the normal centromeres. We do not think that they are such organs. Instead, we shall speak of neo-centric regions.[1]

ÖSTERGREN and PRAKKEN (1946) and RHOADES (1952), working on rye and maize respectively, found that the neo-centric regions were furnished with similar chromosomal spindle fibres as are found at the centromeres. Their observations were made on fixed and stained preparations.

Practically all the previous reports of a neo-centric activity have described this effect for meiosis only. The only paper, in which it was reported for mitotic chromosomes (BOSEMARK, 1957), concerned chromosomes of a special kind at a special stage, *viz.* lagging B-chromosomes at the anaphase of the first pollen grain mitosis.

In the present study we observed the occurrence of a neo-centric activity during ordinary mitosis in several different species. This observation lead us to suppose that the ability to behave in this way may be a normal property of the material of the chromosome arms in general. As a rule, this activity has in our material a very transient character. It usually lasts only a few minutes in each case, and it could be detected only because our study was based on a ciné-film analysis of mitosis in living cells. The duration of the activity is considered in

[1] The fact that we use the expression "neo-centric" in the present paper does not mean that we are completely satisfied with this term. On the contrary, we feel that this expression might be somewhat objectionable, especially in view of the new results. Thus, we think that there is no reason to use a word containing the component "neo". There is nothing to suggest that there is anything new in this effect. Its widespread occurrence, that has now been demonstrated, suggests that it is a normal chromosome phenomenon which like other commonly occurring chromosome features must be supposed to be very old. The reason why we use the expression "neo-centric" in this paper is simply that this term is already established and we do not want to increase the confusion by introducing a new terminology.

more detail below. The previous studies on neo-centric activity reported in the literature were all based on fixed and stained preparations.

This neo-centric activity is the cause of the so-called abrupt bending movements of the chromosomes, a phenomenon discovered by BAJER and MOLÈ-BAJER (1956) and studied in more detail by BAJER (1958 b). The fact that these abrupt bending movements are caused by a neo-centric activity was discovered during the study of ÖSTERGREN and BAJER (1960) on the effects of methanol on mitosis in the endosperm of *Haemanthus katherinae*, and some observations and conclusions concerning this activity were briefly reported in that paper. This study on the neo-centric activity is described in more detail in the present series of papers.

The present paper is devoted to the neo-centric activity found in the arms of ordinary monocentric chromosomes. Another report (ÖSTERGREN and BAJER, 1962) will consider neo-centric movements of acentric chromosome fragments as well as similar effects in bodies other than chromosomes. A discussion of certain theoretical aspects of these effects with a review of related observations from the literature is given by ÖSTERGREN (1962).

MATERIAL AND METHODS

The present investigations are based on studies of mitosis in living cells mainly from the endosperm of various plant species. The method of preparation of the endosperm was described by BAJER (1955) and BAJER and MOLÈ-BAJER (1954). In this method the endosperm cells are spread out on a thin layer of glucose agar (concentration differs according to the species, see the cited papers). Studies with the phase contrast microscope are facilitated by the fact that the cells can be flattened considerably by means of surface tension.

In most cases the material received no special treatment. However, in the case of *Haemanthus katherinae* we used, besides untreated material, endosperm cells that were treated with methanol (ÖSTERGREN and BAJER, 1960). 15 per cent by volume of methanol was added to the agar on which the cells were mounted. As the methanol is diluted with the endosperm liquid that is added with the cells, the effective concentration can be roughly estimated at 3—5 % by volume. The progression of mitosis in the cells was recorded by means of 16 mm ciné-micrography. The number of frames exposed per minute is given below after the name of each material. The films were made by means of phase contrast microscopy, using a microscope from VEB Carl Zeiss of Iena, Germany, with a 90× oil immersion objective and a 5× or occasionally 4× ocular. The cells were photographed on 16 mm negative film, Superpan, of VEB Agfa, Wolfen, Germany.

Evidence for a neo-centric activity was found in endosperm mitosis of the following species: *Haemanthus katherinae, H. albiflos, H. puniceus* (1, 2, 3, 4, 5 frames per min.); *Leucojum aestivum, L. vernum* (8 frames per min.); *Clivia cyrtanthiflora*

(7—9 frames per min.); *Iris aphylla* and *I. pumila* (12 fr. p. m.). Efforts were made to investigate this problem on the endosperm of some other species also, but their chromosomes were too small to be used.

Many of the films from untreated mitoses used in the present study had earlier been used by BAJER and MOLÈ-BAJER (1956) in their analysis of various features of mitosis. Concerning the methanol-treated cells we shall in the present report pay attention only to effects of interest to the problem of neo-centric activity. Other effects of this treatment are described in the papers by ÖSTERGREN and BAJER (1960) and ÖSTERGREN (in preparation). In the present study we made observations also on some cells of *Haemanthus katherinae* which had been treated with β-rays. Our intention was to look for a possible occurrence of the neo-centric activity also in the acentric fragments (ÖSTERGREN and BAJER, 1962). The method of irradiation as well as the more general aspects of mitosis in this treated material were described by BAJER (1958 c). The present observations were made on films used in the previous study of BAJER, and some others. In the present study we also made some observations on certain sequences filmed by BAJER and MOLÈ-BAJER in their studies on the influence of ultra-violet microbeam irradiation on mitosis in *Haemanthus* (BAJER and MOLÈ-BAJER, 1961). The films taken of these cells before their irradiation give us good examples of untreated cells.

Abrupt bending movements, which in our opinion are due to neo-centric activity, were found to be present also in leaf cells of the fern *Hymenophyllum tunbridgense* (film photography, without phase contrast: 10—12 frames per min.). The leaves of this plant consist of a single layer of cells, a fact which makes it relatively useful for observations on mitosis in living cells (HEITZ, 1942; BAJER and MOLÈ-BAJER, 1953).

BLOOM and ZIRKLE have produced an excellent film on mitosis in tissue cultures of the newt *Triturus viridescens*. We have studied their commercially available film from the standpoint of the present problem and in our opinion a neo-centric activity is clearly expressed also in this material.

All photographic illustrations in our present series of papers are based on prints from 16 mm ciné-film negatives. Some of the illustrations are from film sequences that are available in the film "Mitosis in Endosperm II" by A. BAJER and J. MOLÈ-BAJER. In the present papers the illustrations are mirror-images with respect to the way they appear during ordinary projection.

GENERAL ARRANGEMENT OF THE CHROMOSOMES

Before considering the influence of the neo-centric activity on the behaviour of the mitotic chromosomes, it might be appropriate to mention a few words concerning the general arrangement of the chromosome arms during metaphase. This arrangement is produced by factors other than the neo-centric activity and is only temporarily modified by the short periods of this effect.

BAJER and MOLÈ-BAJER (1956), who studied the course of mitosis in living endosperm cells of various plant species, paid some attention also to the behaviour of the chromosome arms. They found that these arms, especially of long and flexible chromosomes, show a strong tendency to become arranged parallel to the spindle axis during prometaphase and metaphase, an arrangement briefly reported already by ÖSTERGREN (1949). As pointed out by BAJER (1958 c), this arrangement of the

chromosome arms is probably caused by the same mechanism which produces the elimination of acentric fragments and nucleolar pieces from the spindle in the poleward directions. These movements of acentric bodies and chromosome arms are according to ÖSTERGREN *et al.* (1960) caused by a pumping activity by means of which the spindle substance works on all sorts of included bodies, striving to move them from the equator towards the two opposite poles during metaphase.

According to the cited interpretation of ÖSTERGREN *et al.*, the arrangement of the chromosome arms at metaphase in plant mitoses of the present kind is caused mainly by the combined influence of two different factors. These are the traction fibres pulling at the centromeres in the two opposite polar directions and the influence of the pumping activity working on the chromosome arms in the same directions. The traction fibres strive to keep the proximal regions of the chromosomes lying flat in the equatorial plane and the pumping activity bends the long chromosome arms near the centromeres and keeps the main part of these arms straight and directed from the equator towards the spindle poles. In the case of small chromosomes the influence of the pumping activity on the arms is less pronounced, with the result that these chromosomes are arranged more or less equatorially along their whole length. This difference in arrangement between short and long chromosomes can be studied in the endosperm cells of *Haemanthus*.

This arrangement of the chromosome arms in the direction towards the spindle poles is characteristic of such mitoses in higher plants in which the chromosomes are long and flexible, as they are in the endosperm cells of the present materials. The same arrangement is also found in the leaf cells of the fern *Hymenophyllum*. In many other mitoses of higher plants, in which the chromosomes are more contracted, they often show a different arrangement with the arms arranged in the metaphase plate or protruding laterally from the spindle equator. This situation was also discussed by ÖSTERGREN *et al.* (1960).

In the newt the chromosomes protrude laterally from the spindle, even though they are very long and flexible. This difference in chromosome arrangement between the newt and higher plants is probably connected with the presence of centrioles in the former material, and with the occurrence of forces causing chromosome arms to move away from these centrioles (ÖSTERGREN *et al.*, 1960). A reference to these so-called "elimination forces" is made later in the present paper in connection with the discussion belonging to Fig. 18.

In endosperm cells of *Haemanthus katherinae* treated with methanol the chromosome arms often show a more equatorial arrangement than in normal cells (ÖSTERGREN and BAJER, 1960; ÖSTERGREN, in preparation). The reason for this difference in arrangement is not quite clear. One possible factor could be a weakening in the treated cells of the spindle forces that in normal cells work on the chromosome arms in the polar directions. Another factor of importance might be the increased occurrence of the neo-centric activity found in the cells influenced by methanol (see below).

Various aspects of the general arrangement and behaviour of the chromosome arms will be discussed again in a paper by MOLÈ-BAJER and ÖSTERGREN (in preparation) which is devoted to a comparative study of the various mitotic movements, with special attention to the non-centromeric type of behaviour.

The metaphase arrangement of the chromosome arms, described above, is temporarily disturbed by the neo-centric movements. Apart from these movements, how-

ever, the chromosome arms also perform movements which may be described as small oscillations. The centromeres remain immobile (or nearly so) in these movements, giving the impression of being anchored in the spindle. The movements are strongest in the distal parts of the arms which are localized in the polar regions of the spindle or even in the cytoplasm. These movements may be related to the Brownian-like movements of small particles that are seen to jump about irregularly in the polar regions of the spindle. The arms also make revolving movements due to the uncoiling of the relational spirals of their chromatids. These oscillating and revolving movements are found also in c-mitosis induced by treatment with colchicine (MOLÈ-BAJER, 1958).

The neo-centric movements differ from these other movements by the fact that they are caused by strictly localized forces of a pulling character. Also, they are usually more rapid and vigorous. Weak neo-centric movements, covering very short distances only, can not always be definitely distinguished from these oscillating movements, however.

NEO-CENTRIC ACTIVITY IN THE ARMS OF ORDINARY CHROMOSOMES

In the absence of a neo-centric activity the chromosomes remain rather stable during metaphase in the arrangement described above. The movements of the chromosome arms during prometaphase which lead to their characteristic metaphase arrangement are also rather slow compared to the movements due to the neo-centric activity that will be described below. These ordinary pro-metaphase movements are also different from the neo-centric movements in so far as the forces producing the ordinary movements appear to be equally distributed over the whole chromosome body (with the exception of the centromere forces, of course). There is no evidence from the movements and the shape of the chromosomes suggesting that localized forces working on the arms would be involved in the ordinary metaphase and pro-metaphase movements. The neo-centric activity on the other hand works very vigorously on small strictly localized chromosome regions (Figs. 1, 2, 3, 4, 6, 7, 10, 11, 12, 14, 16, 17, 18).

The only other process occurring during mitosis which is similar to the neo-centric action is the rapid and vigorous stretching movements of the centromeres occurring when they first become attached to the spindle, an effect described by BAJER (1958 a, pp. 252—253); see also our Fig. 2 C.

A neo-centric activity was earlier observed in fixed and stained preparations from various materials by a number of authors (ÖSTERGREN, 1962). The activity studied in these previous reports must, however,

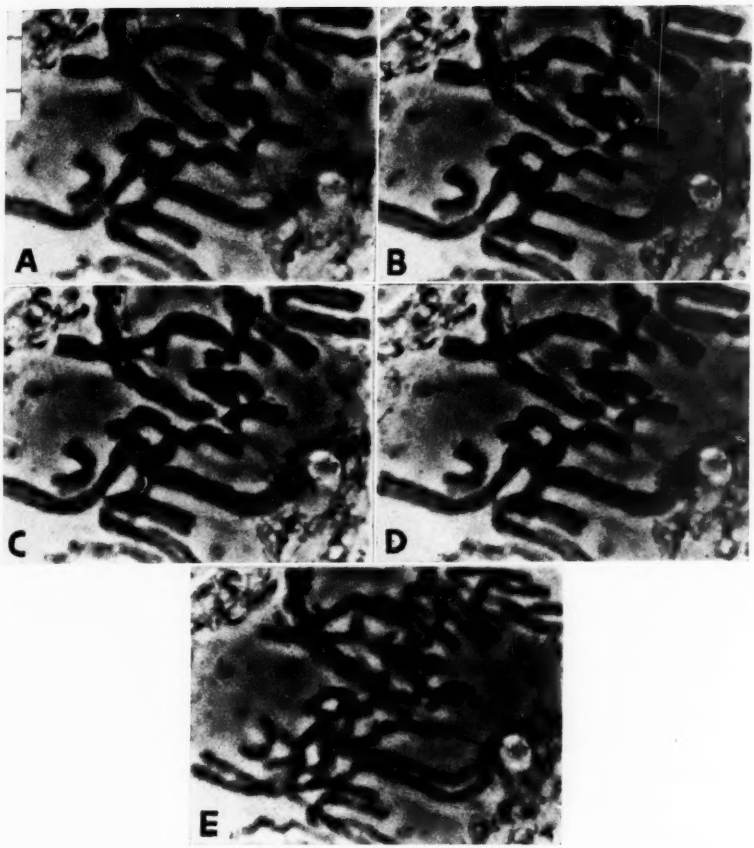
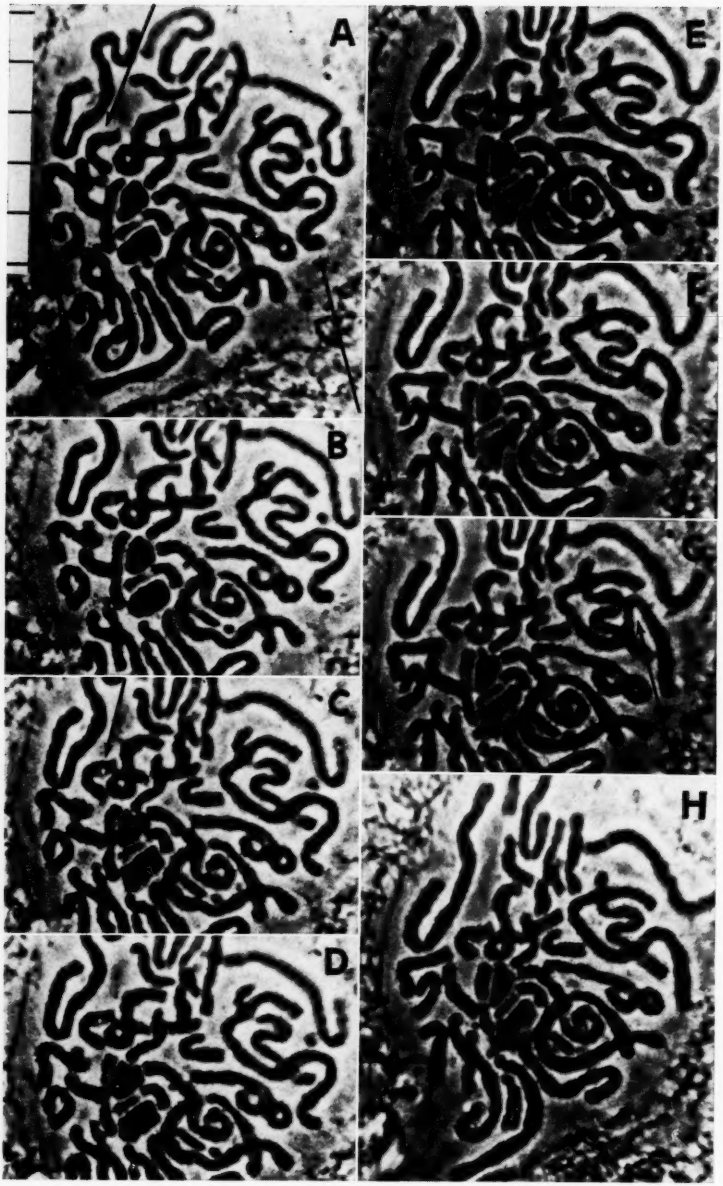


Fig. 1. Neo-centric activity in an untreated endosperm cell of *Haemanthus katherinae*. Two movements of the same chromosome region. — A. Metaphase before the first movement. The chromosome is marked with an arrow. — B and C. During the first movement. — D. Metaphase after the first movement. — E. Early anaphase. The second neo-centric movement. — The spindle equator is diagonally arranged on the figures. As the cell is flattened the centromeres are not all of them completely in the plane of the equator. The long arms have the normal and typical arrangement for such mitoses. They extend from the equatorial centromeres in the directions of the spindle poles. The neo-centric activity appears in one only of the two sister chromatids. (The fibre-like structure, lying across the chromosome, close to the neo-centric region, is a stretched mitochondrion. It is irrelevant to the effect that we are concerned with here.) — The interval on the scale (top left corner) corresponds to 10 μ. — A, 0 min; B, 0 min 30 sec; C, 4 min 40 sec; D, 8 min 30 sec; E, 19 min. — Cell No. 68/59 uv.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have been of a somewhat different character from the effect that we are concerned with in the present study; especially with regard to the duration of the effect that in general must have been longer in each individual instance. Short-lasting neo-centric movements, which are the most common type in our study, would have been very difficult to detect in fixed cells.

The chromosome movements due to a neo-centric activity of the present type were first discovered by BAJER and MOLÈ-BAJER (1956) during their film studies of mitosis in living endosperm cells. They found that the ordinary arrangement of the chromosomes was temporarily modified by the occurrence of sudden bends of the chromosome arms towards the equator, bends that were rapidly followed by a straightening of the chromosomes again (BAJER and MOLÈ-BAJER, 1956, Figs. 6, 7 and 8 pp. 568—570 and pp. 572—575). These bends were studied in more detail by BAJER (1958 b) who called them "the abrupt bending movements". As this type of movement was found to be absent from mitosis treated with colchicine (MOLÈ-BAJER, 1958) it was concluded that these bends were produced by some kind of action of the spindle (BAJER, 1958 b; MOLÈ-BAJER, 1958) but no specific ideas were presented as to the type of spindle action causing these movements.

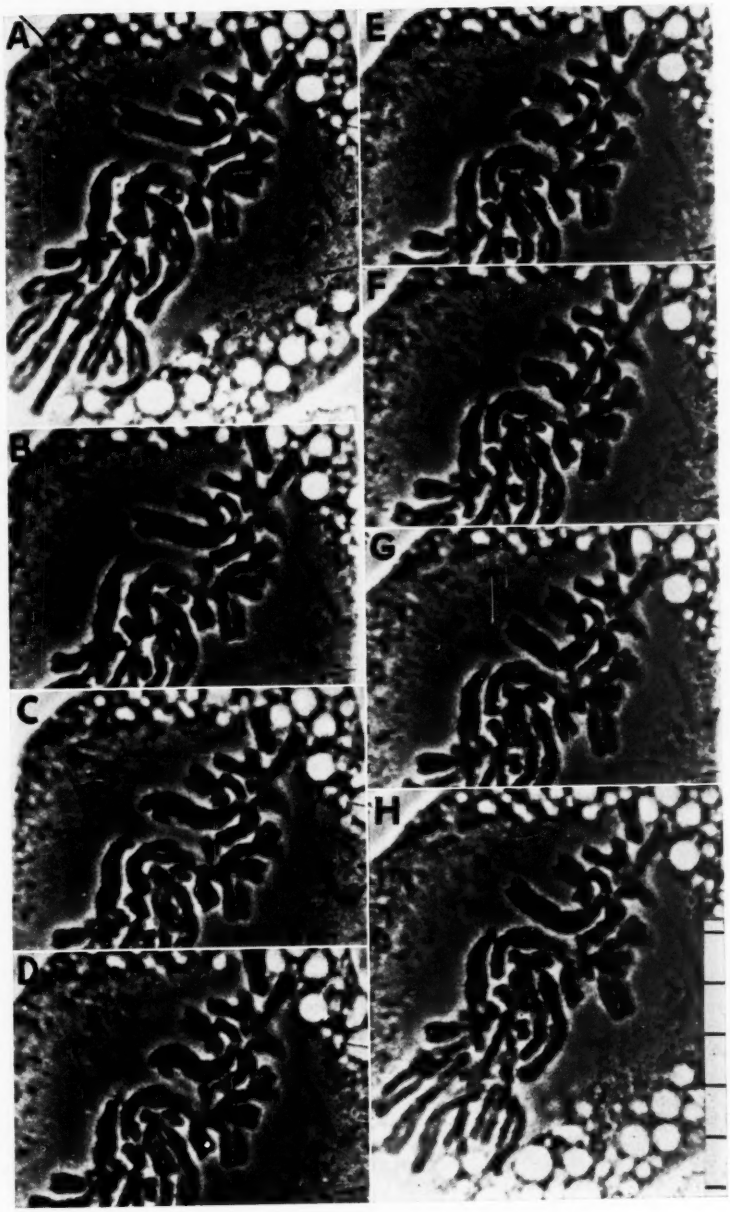
In the present study it was found that these abrupt bending movements are due to an active mobility on the spindle of small localized regions in the chromosome arms. The action of the spindle on these regions is of a type that must be characterized as a pulling one. The chromosomes are elastically deformed by the pulling action, small regions of them are drawn out to points during the movement and these points immediately disappear again, when the pulling has stopped. The pulling force is capable of working in different directions on the spindle. Usually, however, it is directed towards the metaphase plate. The rest of the chromosome arm is passively dragged along by the active region, just as in the case of the centromere movements. This primary movement is followed by a movement in the opposite direction, in which the chromosome returns to its original position.

Fig. 2. Neo-centric activity and centromere stretching at prometaphase in an untreated endosperm cell of *Haemanthus katherinae*. — In Fig. A the two pertinent chromosomes are marked with arrows. — C shows the maximum of the centromere stretching and G the maximum of the neo-centric movement (arrow). — Each interval of the scale=10 μ. — A, 0 min; B, 3 min 1 sec; C, 5 min 12 sec; D, 8 min; E, 10 min 55 sec; F, 11 min 44 sec; G, 12 min 36 sec; H, 15 min 39 sec. — Cell No. 157/57= Cell No. 17 of the film "Mitosis in Endosperm II" by A. BAJER and J. MOLÈ-BAJER.

The discovery that the abrupt bending movements are due to a pulling action of the spindle on small localized chromosome regions was made during a study of methanol-treated mitoses in *Haemanthus katherinae*. The movements and deformations of the chromosomes are more pronounced in this treated material; but, when the phenomenon had been once discovered, it was easy to find the same effect also in the untreated cells of *Haemanthus*, as well as in the endosperm of the other species mentioned in the paragraph on material and methods. The drawn out points of the chromosome material are clearly seen in many mitoses, but there are also many mitoses in which the abrupt bending movements occur without observable drawn out points. The shape of the chromosomes, when they bend, however, supports the interpretation that the bending movements are caused by a pull working on small localized regions also in chromosomes not showing drawn out points.

It is in no way surprising that there is a variation in the visibility or degree of expression of the deformation induced in the chromosome region that is subjected to the pulling force of the neo-centric activity. We cannot find that this variation would justify a suggestion that these abrupt bending movements might be heterogeneous in their origin, that is that they would be due sometimes to a pulling force and sometimes to something else. There is a big variation in the distances covered by the abrupt bending movements, and it is clear that short-distance movements due to weaker forces should be combined with less deformation in the chromosomes. There is also a variation in the consistency of the chromosomes, and when they are softer, the deformations should be larger. There are also technical difficulties arising from the small size of the chromosomes in certain species and the limitations due to the resolving power of the microscope and the photographic emulsion of the film.

Fig. 3. Neo-centric activity in a cell of *Haemanthus katherinae* treated with methanol. The activity appears at metaphase in the end of a chromatid. (The sister chromatid shows no effect.) — The pertinent chromatid is marked with an arrow in Fig. A. The strongest deformation is found in Fig. C. After a decrease of the effect in D, it is again intensified in E (repeated movement). — Note that the chromatid ends that were separated have re-associated in Figs. F—H. In Fig. F the ends are associated in spite of a subterminal separation of the chromatids. The subterminal separation may be caused by a weak neo-centric activity in this region. — The relatively equatorial arrangement of the chromosome arms, seen here, is often found in methanol-treated cells. — The cell had been influenced by methanol for about 3 hrs at the start of this series of figures. — Each interval of the scale corresponds to 10 μ. — A, 0 min; B, 1 min 20 sec; C, 6 min 20 sec; D, 11 min 40 sec; E, 13 min; F, 18 min 40 sec; G, 22 min; H, 25 min. — Cell No. 191Mb/57.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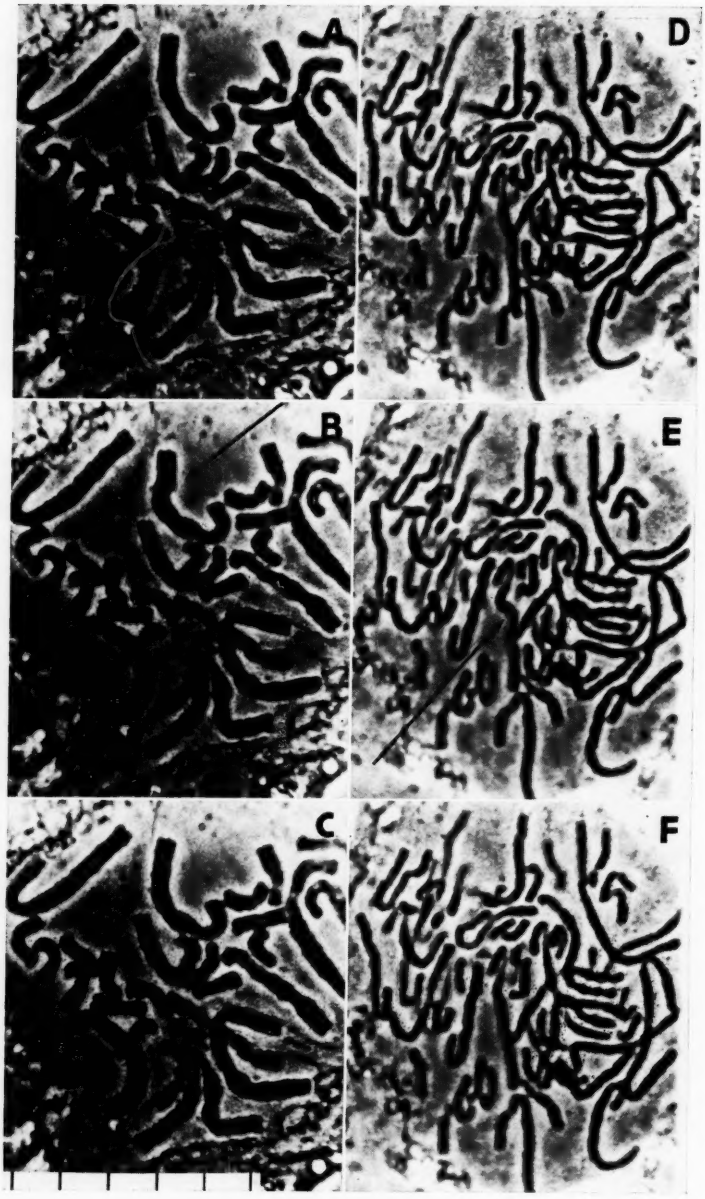
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The neo-centric activity is most easily studied in *Haemanthus*, as this genus has the largest chromosomes among our materials, and the most detailed study of the effect has, as yet, been made on *Haemanthus katherinae*. Table 1 gives a summary of all the visible neo-centric movements in a number of cells of this species. Untreated cells and cells treated with methanol were studied.

Table 1 does not suggest any difference in the frequency of the neo-centric movements between the normal and the treated cells. The total number of such movements noted during 30.60 hrs of analyzed mitosis (prometaphase and metaphase) in methanol-treated cells was 347, while that noted during 32.23 hrs of untreated mitosis was 399. This gives us an average value of 11.34 neo-centric movements per cell per hour in the methanol-treated cells and 12.38 such movements in the untreated cells.

It should be noticed, however, that Table 1 is not based on material that permits comparisons of this kind. The group of untreated cells contained, besides a random sample of such cells, two cells that were especially selected for study because they showed an exceptionally high frequency of movements of this kind. When these selected cells are removed the untreated cells have an average of 6.44 neo-centric movements per cell per hour, which is a value considerably lower than that of the treated cells. Statistical tests of these observations at first failed to demonstrate a clear significance in the difference between the treated and the untreated groups. When attention was paid also to another source of variation in these cells, however, a more significant result was obtained.

Because of technical conditions and differences in their arrangement the chromosomes are not equally well visible in different cells. In order to minimize this source of variation, the analysis was concentrated on the long chromosomes, and the number of well visible long chromosomes was counted for each cell. The number of neo-centric movements of the long chromosomes per well visible long chromosome and per hour of analyzed mitosis was calculated for each cell. The distribution of these figures for the individual cells can be seen in the graph of Fig. 5. The mean value for the untreated cells is 0.81 and for the treated ones 1.36. The two exceptional cells of the untreated group are not included.

The distribution of the cell values of Fig. 5 was tested by means of a χ^2 test. The

Fig. 4. Neo-centric activity at endosperm mitosis in *Haemanthus katherinae*. — Figs. A—C. Untreated cell at metaphase. — Figs. D—F. Very weak methanol-treatment; cell at anaphase. — A and D. Before the effect. — B and E. During the neo-centric movement. The neo-centric regions are marked with arrows. — C and F. After the movement. — The neo-centric region in E moves in the same direction as the centromere, but the neo-centric movement is more rapid. — The irregular shape of the metaphase plate in Figs. A—C is due to the flattening of the cell. In spite of this irregularity a bipolar anaphase movement occurred. — Each interval of the scale = 10 μ. — A—C. Cell No. 157/57 = Cell No. 17 of the film "Mitosis in Endosperm II" by A. BAJER and J. MOLÈ-BAJER. A, 0 min; B, 3 min 11 sec; C, 4 min 21 sec. — D—F. Cell No. 158M/57. D, 0 min; E, 1 min 15 sec; F, 3 min 15 sec. Methanol treatment of the cell *ca* 1 % for 10—11 hrs.

cells were divided into two groups; those having a value higher than 0.63 were placed in one group and those having a value lower than this limit were placed in a second group. The limit 0.63 was chosen because half the number of the cells in the total material were below this limit and half of them were above. The test strongly suggests that there is a difference between treated and untreated cells, the p-value being between 0.02 and 0.01.

Thus, we find that there is some evidence for the opinion that the methanol treatment has an influence on the frequency of the neo-centric movements in this material. The main effect, however, of the methanol treatment on the neo-centric movements is another kind, *viz.*

TABLE 1. *Instances of neo-centric action in untreated and in methanol-treated cells of Haemanthus katherinae.*

The chromosome number in the endosperm cells is 3n=27. There are 12 long chromosomes belonging to 4 types and 15 short chromosomes belonging to 5 types. In each instance only the primary movement is recorded. This is followed by a return towards the original position. Repeated movements are recorded separately.

Part A. *Number of cells and total time of filming of the analyzed sequences.*

	Number of cells	Total time, hrs
Untreated	21	32.23
Methanol-treated	13	30.60

Part B. *Observations on the long chromosomes.*

	No. of well visible long chromosomes	No. of long chromosomes showing neo-centric action	Number of movements directed:			Location in the chromosome:	
			Towards the plate	Transversely or obliquely to the spindle	Towards the nearest pole	Terminal	Interstitial
Untr.	221	96	317	7	2	52	274
Meth.	131	85	267	24	7	41	257

Part C. *Observations on the short chromosomes.*

	No. of short chromosomes showing neo-centric action	Number of movements directed:			Location in the chromosome:	
		Towards the plate	Transversely or obliquely to the spindle	Towards the nearest pole	Terminal	Interstitial
Untr.	13	67	4	2	39	34
Meth.	22	41	8	—	40	9

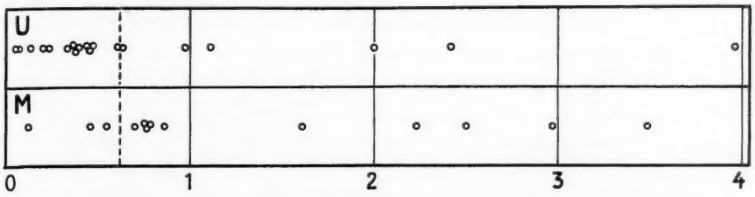
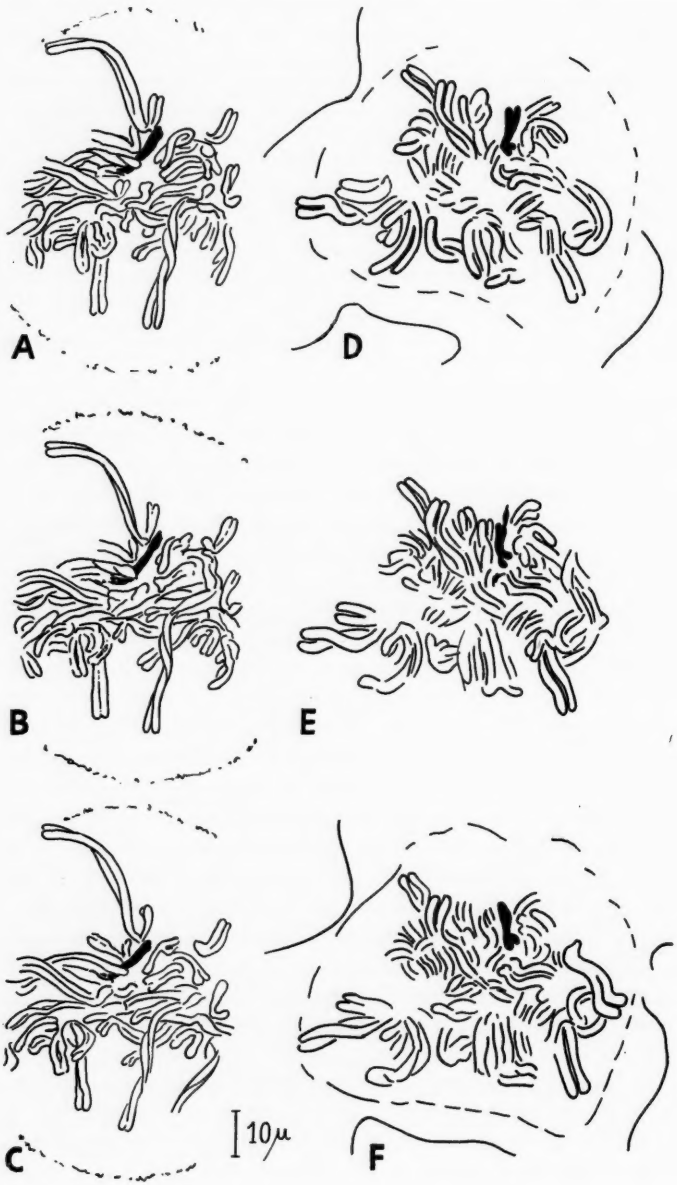


Fig. 5. The number of neo-centric movements of the long chromosomes per well visible long chromosome per hour of film recording. Each dot corresponds to a nucleus in mitosis (prometaphase and metaphase). U=untreated material. M=methanol-treated material. The dotted line corresponds to the value 0.63. Half the number of nuclei of the total material are above and half are below this limit. The distribution of the untreated nuclei is 13:6 and of the methanol-treated ones 3:10. This gives $\chi^2=6.35$ and p between 0.02 and 0.01. Material: Endosperm of *Haemanthus katherinae*.

it is an influence on the *expression* of the effect rather than on its frequency. In the treated cells the neo-centric force often causes a stronger deformation of the chromosomes; the drawn out points where the pull is working on the chromosomes are often more pronounced. This may be due to a softer consistency of the chromosomes in the treated cells (ÖSTERGREN and BAJER, 1960), or to the forces of the neo-centric action being stronger, or to both.

A big variation was found when comparing different cells in the untreated material. We have already mentioned that two of the cells showed an exceptionally high frequency of neo-centric movements. It is obvious that the fact that the cells are untreated does not prove that they are normal. The cells have been removed from their normal environment in the ovule and mounted on an artificial medium of glucose agar. This may influence them in different ways. Metabolic disturbances or stimulations might provoke various responses. It is quite possible that the neo-centric activity in the untreated cells might be caused by influences of this kind. Even if all the neo-centric activity in the untreated cells were of this induced kind, it would nevertheless be a very interesting effect in demonstrating the reaction potentialities that exist in the chromosomes.

As can be seen from Table 1 the great majority of the movements are directed towards the metaphase plate (=the spindle equator). Instead of saying that the movements are directed towards the metaphase plate we might also say that they are directed towards the more remote spindle pole. When speaking of the *direction* of a neo-centric movement, we mean the direction of the primary movement. This is followed by a return movement in the opposite direction.


A
D
B
E
C
10μ
F

Movements towards the nearest spindle pole are also found, although they are much more rare. As they are so rare, we have, however, used special care to check that they really exist. We have found, both from observations of the cells analyzed in Table 1 and from many other cells, that, without the slightest doubt, neo-centric movements exist that are directed towards the nearest pole. Although these movements usually are weaker than those of the opposite type, they can also in some cases be quite strong (Fig. 6).

In most of the neo-centric movements the pull producing them obviously works along the fibre structure of the spindle. There are a few cases that are less clear in this respect. These can, however, in a more indirect way be given the same interpretation (*e.g.* Fig. 7).

It is true, that the fibre structure of the spindle practically is not visible at all in the living cells with which we are concerned in the present study. The direction of the fibres in cells of this kind is known to us, however, from four sources of information:

(1) In the studies of INOUÉ and BAJER (1961) and BAJER (1961) the chromosomal spindle fibres of the centromeres could be very well demonstrated in the endosperm of *Haemanthus* by means of polarized light.

(2) In some living cells in this material these centromeric spindle fibres are weakly but distinctly visible also in ordinary phase contrast pictures. Visibility of the spindle fibres is usually considered to be a symptom of an abnormal state in a cell, and it is probable that this may be so in our cells, too. As, however, mitosis in these cells develops normally from prophase to telophase, the degree of abnormally must be very small.

Fig. 6. Endosperm cells of *Haemanthus katherinae* showing neo-centric movements directed towards the nearest spindle pole. — Figs. A—C. Cell No. 158M/57, given a very weak methanol treatment. — Figs. D—F. Cell No. 180M/57, given the ordinary kind of methanol treatment. — Figs. A and D. Before the strong movement, but probably with a weak activity here, too. — Figs. B and E. During the movement. — Figs. C and F. After the movement. — Drawings made from ciné-film projections. The pertinent chromosomes black, the others only approximately indicated. — In the chromosome of Figs. A—C there were neo-centric movements already before Fig. A. The chromosome end made a series of small but distinct movements in the polar direction during the major part of prometaphase and early metaphase, a behaviour most probably corresponding to a very long-lasting (*ca* 55 min) though weak activity of a variable intensity, the periods of higher intensity corresponding to the polar movements. — In the chromosome of Figs. D—F the chromatid in question made an abrupt bending movement towards the plate before Fig. D; it is probably weakly active towards the pole in D and shows a long-lasting (20—25 min) activity of variable intensity in the polar direction. A moment of high intensity is illustrated in E. — The two cells of Fig. 6 may show us instances in which neo-centric regions of a long-lasting weak activity become stabilized in a polar orientation through a co-orientation of these regions relative to the centromeres of the same chromosomes (see, ÖSTERGREN, 1962). — The deviation from a perfect equatorial arrangement of the centromeres seen in Figs. A—C is caused by the flattening of the cell, and the irregular chromosome arrangement in Figs. D—F is a methanol effect.

(3) The course of the fibres in mitoses of higher plants in general is known to us from observations of fixed material.

(4) The movements of the anaphase chromosomes follow curved paths that appear to agree essentially with the course of the spindle fibres.

When the direction of the neo-centric pull working on the chromosome arms is compared with the information from these sources it is found that it agrees reasonably well. It would be interesting to know whether or not chromosomal spindle fibres are attached also to the neo-centric chromosome regions in the endosperm cells. In a few cells photographed with phase contrast such fibres are faintly indicated.

Table 1 describes a number of neo-centric movements as "transverse or oblique" because the neo-centric regions performing these movements did not move directly either towards the nearest pole or towards the equator. In many of these cases a closer examination shows the *pull*, itself, to be directed along the fibre structure, although the *movement* is performed in another direction because the firm attachment of the centromere to the spindle prevents a movement in the immediate direction of the pull. An example of this phenomenon is given in Fig. 7 where a chromosome end is moved in a direction that is practically parallel to the metaphase plate, as the result of a pull working in the direction towards the nearest pole. The centromere of the chromosome is firmly attached in the metaphase plate and the chromosome arm executes a temporary pendulation in the direction towards the centre of the plate.

A very reasonable interpretation of some movements cross-wise to the spindle fibres is expressed in the hypothesis that the neo-centric region is attached to spindle fibres pulling in both the opposite poleward directions. If these two pulling forces are not directly opposed, a resultant force will be produced, working in a more or less transverse direction (see, Fig. 18 D, and the figures of ÖSTERGREN, 1962).

The data of Table 1 show a significantly higher frequency of "transverse or oblique" neo-centric movements in the methanol-treated cells than in the untreated material. A χ^2 test gives a p value less than 0.001. This difference is well explained by the disorganizing action of the methanol treatment on the polarity of the spindle. This treatment very often causes multipolar spindles or even, as an extreme effect, a nearly complete c-mitosis (ÖSTERGREN and BAJER, 1960). It is easy to see that the disturbed arrangement of the spindle fibres can give the impression that more movements than in normal cells take place transversely in the spindle, although, in reality, even these movements are taking place along the (disarranged) fibre structure of the spindle. Many of the

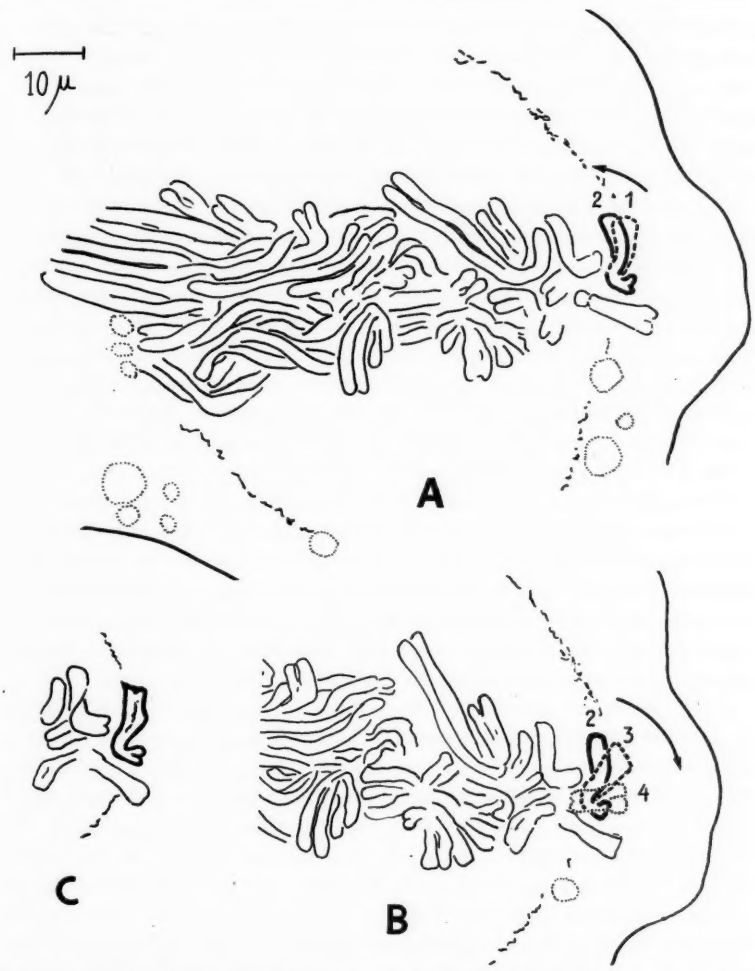


Fig. 7. A methanol-treated endosperm cell of *Haemanthus katherinae* showing a short-lasting neo-centric movement transverse to the spindle. — A. The "foreward" movement from 1 to 2. — B. The "return" and later movements from 2 to 3 and 4. — C. At the moment of maximum force (between 1 and 2) the pulling mechanism deforms the chromosome end, causing a small pointed tip, directed towards the nearest spindle pole. — The obvious interpretation is that the transverse movement is caused by a force working towards the nearest spindle pole. The return movement and the later movement probably result from relaxation from an elastic deformation combined with the so-called "elimination forces" of ÖSTERGREN *et al.* (1960). These "elimination forces" act to move chromosome arms out of the organized spindle region. — Cell No. 191Mb/57.

methanol cells used in Table 1 had multipolar spindles or showed disturbances in the metaphase arrangement of the chromosomes.

In most cases reported in the literature the neo-centric activity was located terminally or sub-terminally on the chromosomes (ÖSTERGREN, 1962). Our own observations on the location of the neo-centric regions in the *Haemanthus* chromosomes are summarized in Table 1.

Both the interstitial and the terminal location of the activity are of common occurrence. Generally speaking, neo-centric regions are capable of occurring in any position along these chromosomes from close to the centromeres to the ends of the arms. In the long chromosomes, interstitial locations are found to be about five times more common than terminal. However, in the short chromosomes the relative proportion of terminal locations is much higher. A difference between long and short chromosomes of this general character is, of course, to be expected from the simple fact that the interstitial region is much shorter in the short than in the long chromosomes.

At first glance our data might seem to support the opinion that there is a certain preference for a terminal location of this activity also in our material, the chromosome ends showing this behaviour more often than interstitial regions of the same small size. A certain cautiousness is appropriate, however, in making conclusions from our material in this respect, and we think that it may not be justified to express a definite opinion on this question from the present results.

Thus, it should be realized that there is probably a much greater chance for a terminal activity to be visibly expressed because the chromosome ends are lying in a freer position than much of the chromosome body, which is often crowded with the other chromosomes in the plate. Factors of this kind might explain the statistically significant difference ($p<0.01$) that is found in the short chromosomes of Table 1 between untreated and methanol cells with respect to the relative proportion of terminal and interstitial cases. In the methanol cells the body repulsion between the chromosomes appears to be decreased (ÖSTERGREN and BAJER, 1960) resulting in increased crowding of the chromosomes and a decreased chance of observing interstitial cases of neo-centric activity. Furthermore, it should be realized in connection with the general question of the relative proportion of terminal and interstitial cases that the exact location of the neo-centric region cannot always be exactly defined and that for this reason some sub-terminal locations might have been recorded as "terminal".

When the neo-centric activity results in a pulled out (pointed) region

of the chromosome material, it can be seen that in each instance the activity works in one only of the two chromatids of a metaphase or prometaphase chromosome (*e.g.* Fig. 1). This independence of the two chromatids with respect to the appearance of the neo-centric activity is clearly expressed at late metaphase, when the sister chromatids are relatively more free of each other. Then it can often be seen that the active movement is performed by a single chromatid only. In its movement this chromatid separates widely from its sister chromatid which is only weakly and passively dragged along by the relatively weak chromatid association. In prometaphase and early metaphase, when the chromatid pairing is more firm, the chromatids follow one another much more closely in their movements.

It is often found that the same chromosome executes a series of successive neo-centric movements. Then, the neo-centric activity usually appears in different regions along the chromosome length, and it works sometimes in the one and sometimes in the other of the sister chromatids. Two instances of repeated movements in which the activity changed from one chromatid to the other are represented in the two lower graphs of Fig. 9. For one of these instances pictures of the chromosome are drawn in the graph to show the changes in its shape during these movements. The activity in this case appeared to be terminally located; however, no drawn out points of chromosome material were observed. Another case of repeated neo-centric movements is illustrated by the photographs of Fig. 10.

Sister chromatids separated by neo-centric movements usually re-associate with one another more or less closely when the neo-centric action stops. We are unable to judge conclusively, at present, whether this re-association is due merely to an elastic straightening of the chromatids that remained associated in other parts, or whether real pairing forces between homologous chromatid regions might be involved. Such a case as that photographed in Fig. 3 gives considerable support, however, to the idea that real pairing forces might be involved. After the ends of the chromatids were separated by the neo-centric action in Figs. 3 C—E, they were again closely associated in Figs. 3 F—H in spite of the fact that the sub-terminal chromatid regions now show some separation. The sub-terminal separation was presumably caused by a weak neo-centric activity that appeared in this region. Naturally, such evidence for pairing forces does not in itself give us any information concerning their nature. They might be due to thinly stretched connections of matrix material (JACOBSEN, 1960) or to some other mechanism.

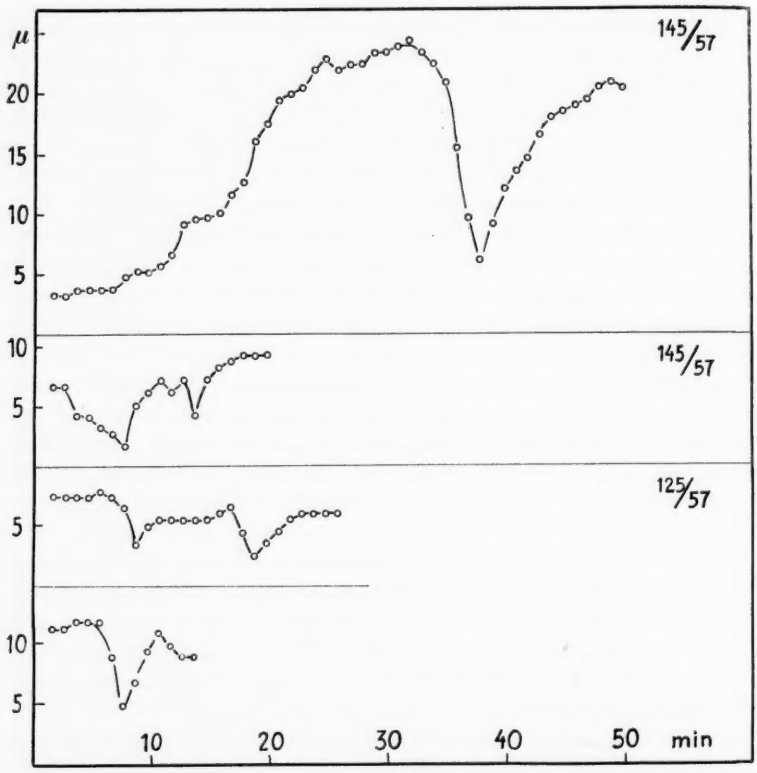


Fig. 8. Graphs of neo-centric movements of chromosome ends at metaphase in untreated endosperm cells of *Haemanthus katherinae*. — Ordinate: Distance in μ of the chromosome end from an arbitrarily chosen level approximately corresponding to the spindle equator. The distances are measured perpendicular to this level. — Abscissa: Time in minutes. — The beginning of the upper curve corresponds to the prometaphase straightening of the chromosome arm in the direction towards a spindle pole. — Cells No. 145/57 and 125/57.

It is possible that even smaller sub-units of the chromosomes than the chromatids may be able to show separate neo-centric activity. This is suggested in Fig. 11, in which a pulling action on a single half-chromatid appears to open a temporary hole between the two half-chromatids.

Neo-centric activity is found at anaphase as well as at prometaphase and metaphase. A table showing its frequency at these different stages

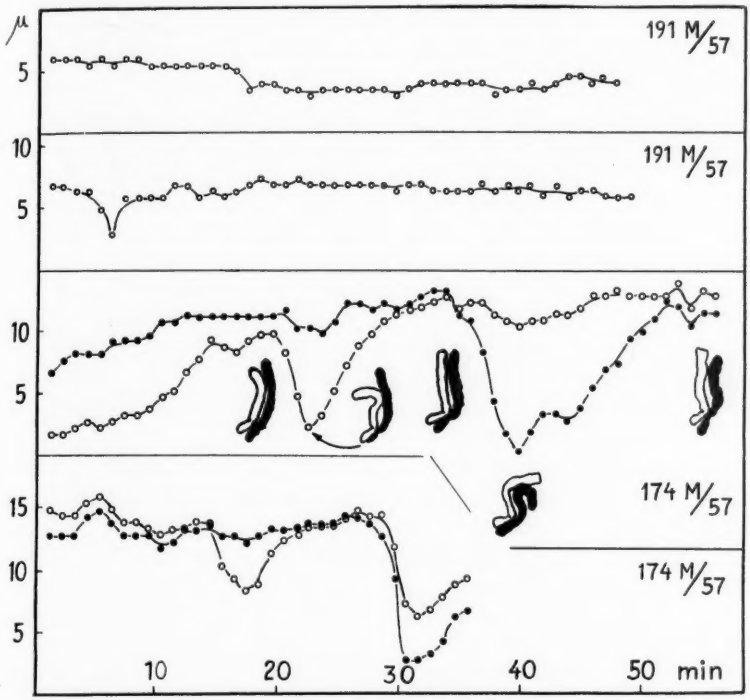


Fig. 9. Graphs of neo-centric movements of chromosome ends at metaphase in methanol-treated endosperm cells of *Haemanthus katherinae*. — The movements are plotted as in Fig. 8. — In the curves made from two different chromosomes of cell No. 174M/57 the two sister chromatids of each chromosome make separate movements. These chromatid movements have been plotted individually side-by-side, one chromatid being represented by white and one by black dots. One of these double curves is accompanied by drawings showing the changes in shape of the chromosome. — Cells No. 191M/57 and 174M/57.

was presented by BAJER (1958 b, p. 377). No new studies have been made in the present work concerning its frequency at different mitotic stages.

In the paper of BAJER (1958 b) the opinion was expressed that the abrupt bending movements in *Haemanthus* at anaphase were different in different regions of the anaphase spindle. In the half spindles the first stage of the movement was directed towards the equatorial plane. On the other hand, the primary movement in the interzonal region ap-

peared to be in the poleward direction. A careful re-investigation of this question has now demonstrated that this opinion was wrong.

The neo-centric movements in these endosperm cells have the same character at all stages and in all spindle regions. In the interzonal region of the anaphase spindle, just as at the other stages, the great majority of the movements are during their first stage directed towards the spindle equator. This first movement, which is caused by a neo-centric pull towards the equator, is followed by a return movement directed towards the previous position of the chromosome.

The neo-centric movements in *Haemanthus* may be further characterized by the following information concerning their *duration*, *speed*, and *distance* of movement. Furthermore, the relative *size of the forces* producing them may be roughly estimated. The movements are very rapid. They are actually the most rapid of all the chromosomal movements found in these cells. In its most rapid phase the speed often reaches the order of 5 μ/min. The anaphase movements have a maximum speed of less than 2 μ/min. It can be concluded from the deformations they cause in the chromosomes that the neo-centric forces often are very strong. They are often quite comparable to the centromere forces that stretch dicentric chromosome bridges in anaphase.

The reason why these neo-centric forces do not upset the regular distribution of the chromosomes to the daughter nuclei or cause chromosome breakage is chiefly due to their short duration. The total duration of the two movements of which a neo-centric movement is composed (forward and backward) often amounts to about 3—6 min. It seems that very many of these movements may actually be of even much shorter duration, and inconspicuous because of their very short duration and the small distance they cover.

Some neo-centric movements, however, may last for longer periods. There is probably no definite upper limit for their duration except the duration of mitosis itself. The longer they last, the more rarely do they occur, however. The instances illustrated in Fig. 6 most probably represent two cases of very long lasting activity (namely, 20—25 and *ca* 55 min, respectively). In these cases, too, the normal distribution of the chromosomes was but little influenced, but this was not due to the short duration of the activity. In these two instances the slight influence on the chromosome arrangement is a consequence of the weakness of the neo-centric forces.

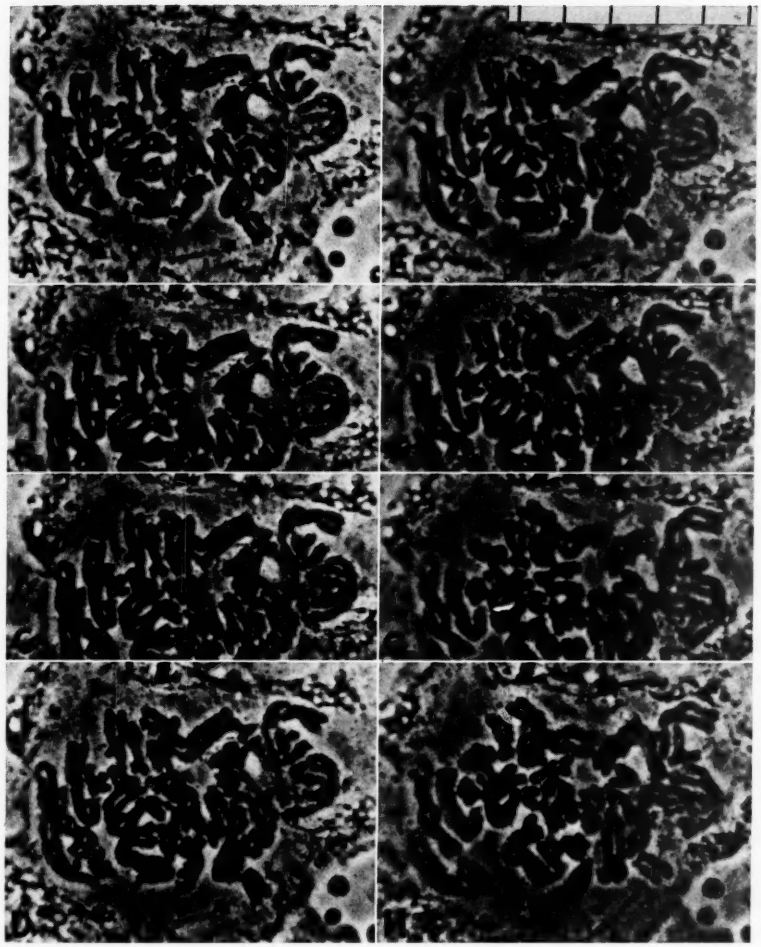


Fig. 10. Neo-centric activity in methanol-treated material of *Haemanthus katherinae*. Repeated movements in various interstitial positions of a chromosome arm (marked with an arrow in Fig. A). — Maximum of one movement in C and of another in F. The chromosome made a series of successive movements, and they are not all included in this series of pictures. — This very irregular metaphase arrangement of the chromosomes is often found in methanol-treated cells. The cell had been treated for about 8 hrs in Fig. A. — Each interval of the scale corresponds to 10 μ. — A, 0 min; B, 4 min; C, 6 min; D, 10 min 30 sec; E, 18 min; F, 29 min 30 sec; G, 60 min; H, 73 min 30 sec. — Cell No. 180M/57.

The weakness of the pulling force in these cases might be due to the fact that the force was directed towards the nearest pole. As pointed out by ÖSTERGREN (1945 a, 1950, 1951), ÖSTERGREN and PRAKKEN (1946), ÖSTERGREN *et al.* (1960), the force of the traction fibre pull grows stronger with an increased distance between centromere and spindle pole. This rule which explains why metaphase centromeres stabilize in the equator, also gives us a natural explanation for the fact that the great majority of the neo-centric movements in the present study are directed towards the spindle equator, ÖSTERGREN (1962).

The direction of the activity towards the nearest spindle pole in the two chromosomes of Fig. 6 may be associated with the long duration of the activity. When the neo-centric activity is more long-lasting, there is an increased probability for the neo-centric region to stabilize in a poleward orientation. This stabilization is the consequence of a co-orientation of the neo-centric region relative to the centromere of the chromosome (ÖSTERGREN, 1962).

When we are concerned with effects of what appears to be a long-lasting neo-centric activity, it is difficult to decide whether the effect is due to a continuous or an interrupted and repeated activity. The impression of the chromosome behaviour illustrated in Fig. 6 is that of a continuous long-lasting activity of variable intensity. During most of the time the activity is weak. However, there are shorter periods of increased intensity corresponding to the neo-centric movements in the polar direction.

The chromosome considered in Fig. 10 made a series of several neo-centric movements in which the activity appeared in neighbouring positions on the chromosome arm. They were all directed towards the metaphase plate. The reason why the chromosome did not approach the equator more closely may be due to the restricted mobility of its proximal region, caused by the crowding of the chromosomes combined with the flattening of the cell.

Instances, in which a series of successive neo-centric movements occur in the same chromosome, are not unusual.

The figures given above concerning the duration of the neo-centric activity can be properly appreciated only when compared with the duration of the mitotic stages in the same kind of cells. The following mean values from 12 normal endosperm cells of *Haemanthus katherinae* may be cited from the paper of MOLÈ-BAJER (1958). The duration of prometaphase was 110 min, of metaphase 45 min. The time from the start of anaphase until the start of cell plate formation in telophase was 97 min. The duration of the movement phase of anaphase was found by BAJER (1954) to be 30 to 45 min.

The distances covered by the neo-centric movements vary from very short distances in the very short-lasting movements to distances of

about 15 μ or more in some of the longest. For comparison it may be mentioned that the distance which the centromeres separate from one another during the anaphase movement is of the order of 60 μ (BAJER, 1954, Fig. 8, p. 395).

As already mentioned, there are differences in the size of the distances covered by the neo-centric movements. Some of these distances are longer, a fact which makes the movements more easy to study. The above numerical data concerning size and speed are derived from such movements. There is, however, a gradual transition in length of these distances from relatively long ones to those so short that the movements are barely noticeable at all. The frequency of these short-distance movements is, of course, very difficult to judge. The general impression is, however, that movements covering very short distances may actually be much more common than those of longer distances. Most probably very many movements are of such small dimensions that they cannot be detected at all. This impression, of course, makes all estimates of the frequency of the neo-centric movements appear very uncertain. Furthermore, it should be realized that neo-centric movements over very short distances are difficult to distinguish from the oscillation movements of the chromosome arms. These oscillations are very probably related to the random Brownian-like movements performed by small particles in the cytoplasm and in the polar regions of the spindle. It appears highly probable that these oscillations are very different in their fundamental nature from the neo-centric movements.

In Table 1 only quite clear neo-centric movements were recorded and many such movements of small dimensions and uncertain character were omitted.

As only the most conspicuous movements were considered and the true character of the "abrupt bending movements" was not understood at that time, the estimates by BAJER (1958 b, p. 377) of the frequencies of these movements at different mitotic stages must be considered to be much too low. Nevertheless, the general character of the difference between the stages is, in our opinion, still valid, *i.e.*, the neo-centric movements decrease in their frequency towards full metaphase and increase again in anaphase.

Figs. 8—9 give a graphical representation of some neo-centric movements in untreated and in methanol-treated cells. Instances of terminally located activity were chosen, and the distances of the chromosome ends from the equatorial plane have been plotted against the time.

Fig. 12 shows a very instructive case of a neo-centric movement in an

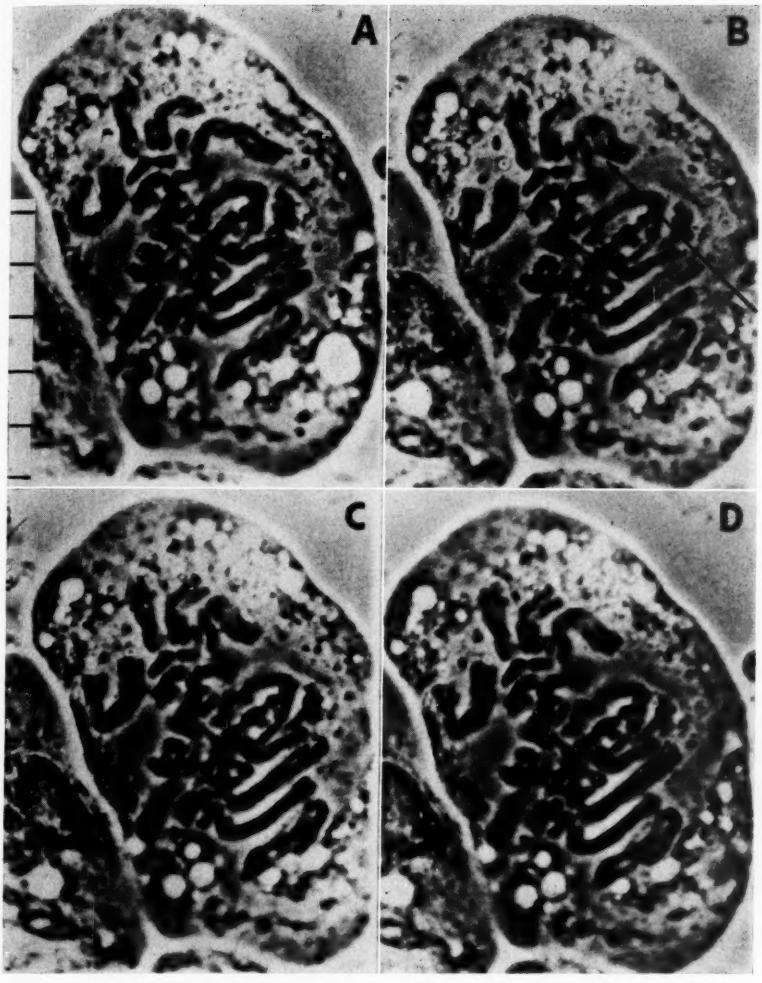


Fig. 11. Neo-centric activity at metaphase in an interstitial chromosome region of a methanol-treated cell of *Haemanthus katherinae*. When the chromosome deformation is strongest (arrow in Fig. B), a small hole appears in the influenced chromatid, a fact suggesting that the activity is restricted to a single half-chromatid only. This chromosome also made neo-centric movements other than the illustrated one. The irregular arrangement of the chromosomes in this cell, some of them outside the metaphase plate, is an effect often found in methanol-treated cells. The cell had been treated with methanol for about 7 hrs when these illustrations were made. — Each interval of the scale=10 μ. — A, 0 min; B, 5 min 20 sec; C, 8 min; D, 9 min 20 sec. — Cell No. 186Mb/57.

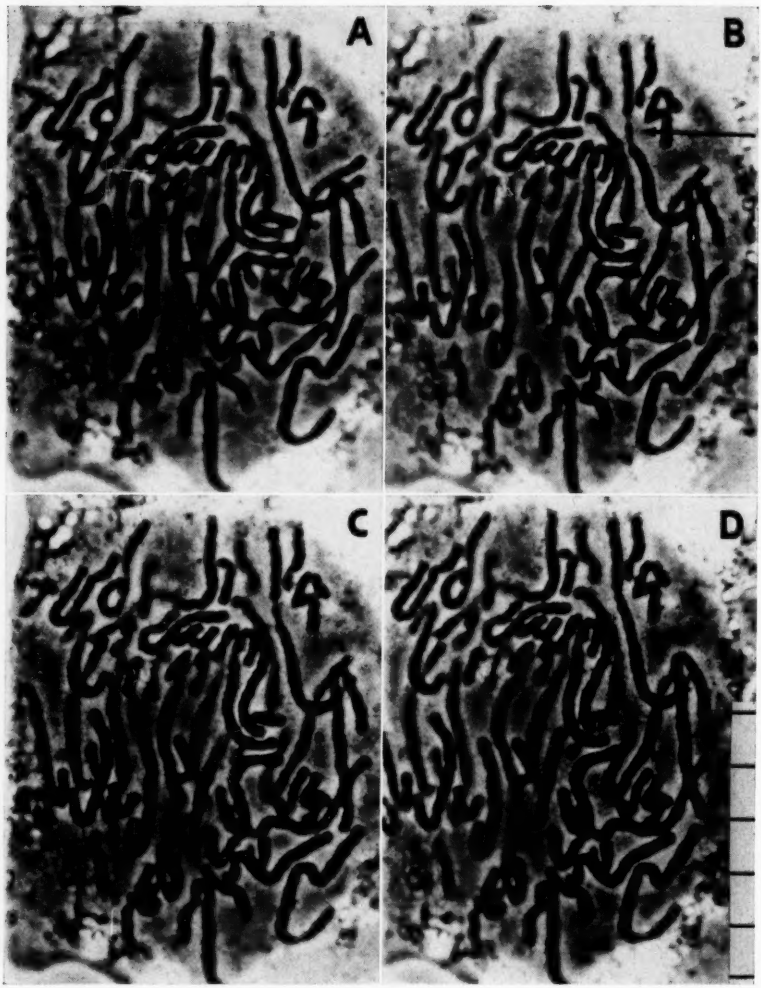


Fig. 12. An endosperm cell of *Haemanthus katherinae* showing a neo-centric movement directed towards the spindle equator at anaphase. A small region in a chromosome arm close to the centromere starts to move rapidly in a direction opposite to the centromere movement (arrow in Fig. B). The chromosome part between the centromere and the neo-centric region is stretched to a thin thread but it immediately re-contracts after the cessation of the neo-centric activity. This behaviour shows that the chromosomes are highly elastic. Traces of lasting deformation in the chromosome indicate that the chromosomes not only show elasticity but also have plastic properties. A graph of these movements is given in Fig. 13. — The cell had been given a very weak methanol treatment (*ca* 1 % for 10—11 hrs). It is practically an untreated cell. — Each interval of the scale=10 μ. — A, 0 min; B, 2 min 30 sec; C, 3 min 15 sec; D, 4 min 44 sec. — Cell No. 158M/57.

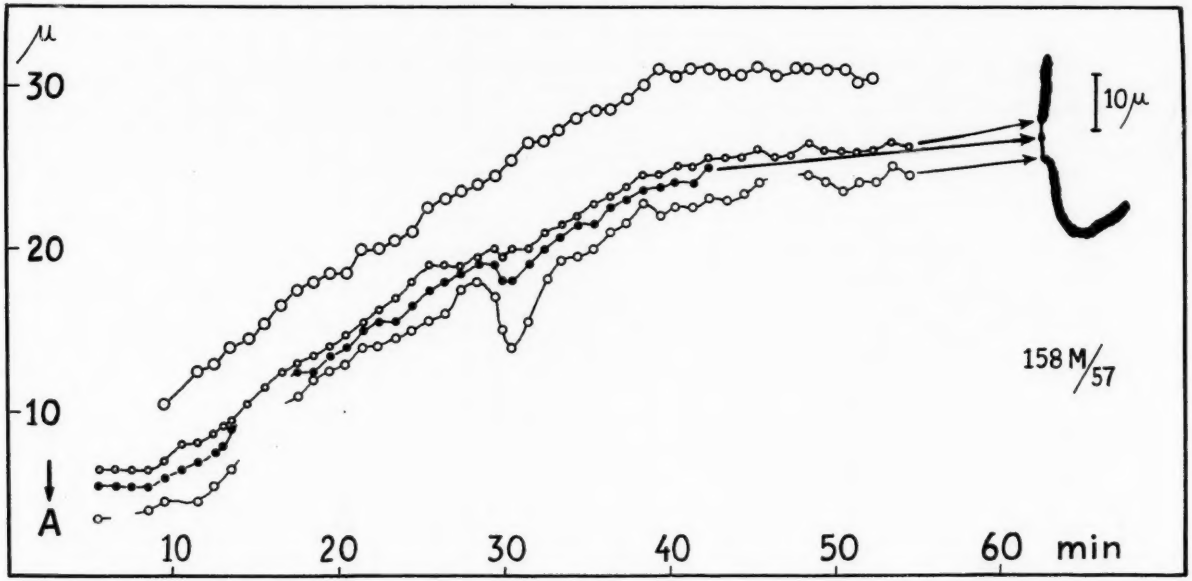


Fig. 13. A graph of some features of the anaphase movements in the cell of Fig. 12. The distance in μ (measured from an arbitrary level, approximately corresponding to the spindle equator) is plotted against the time in minutes. The beginning of anaphase is marked with the arrow A. The top curve shows the centromere path of a chromosome not influenced by neo-centric activity. The three lower curves are drawn from the chromosome showing neo-centric activity and they correspond to the paths of the centromere and two other regions. These regions are marked on the diagram of the chromosome to the right. This chromosome picture is drawn to a much smaller scale than is used in the movement graph. The picture shows the chromosome in the state of maximum stretching. The plotted landmarks of the chromosome are faintly visible also before the neo-centric movements. There are two smaller neo-centric movements before the large one. The centromere movement is temporarily retarded by the neo-centric activity.

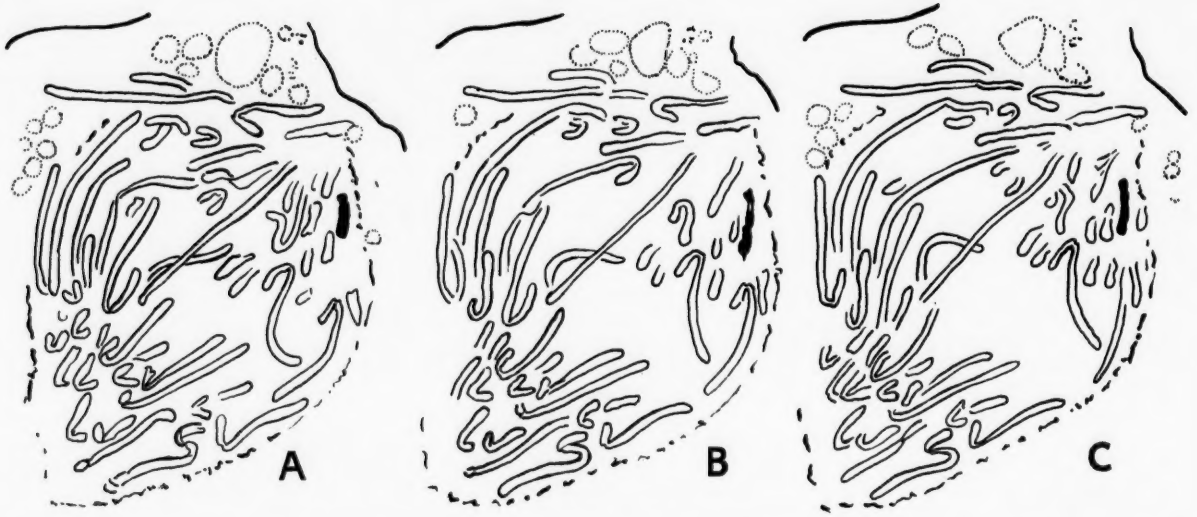


Fig. 14. Neo-centric activity in a chromosome end at late anaphase of endosperm mitosis in *Haemanthus katherinae*. — A. The chromosome has reached the end of its ordinary anaphase movement. (See also the graph of Fig. 15.) — B. The neo-centric force acting towards the spindle equator stretches the chromosome and pulls its centromere away from the pole. — C. After cessation of the activity the centromere returns to its previous position. There remains an increased chromosome length, showing a plastic deformation. — The details of these changes can be seen more clearly in the graph of Fig. 15. — This cell is from β-irradiated material and contains some bridges and acentric fragments besides normal chromosomes. Cell No. 164β/57.

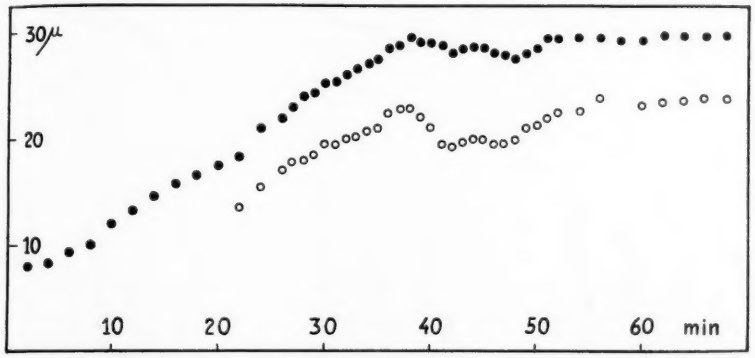


Fig. 15. A graph of the anaphase movement and the neo-centric action from the chromosome of Fig. 14. The distance from an arbitrary level (approximately corresponding to the spindle equator) is plotted against the time. The top curve shows the path of the centromere and the bottom curve that of the chromosome end. Further comments are given in the legend to Fig. 14.

anaphase chromosome. A region close to the centromere is suddenly pulled in a direction opposite to that of the centromere movement, and the small chromosome piece between the centromere and the neo-centric region is strongly stretched. The stretching is quite comparable to that of dicentric bridges at anaphase. This instance is graphically represented in Fig. 13. It can be seen that the poleward movement of the centromere is weakly and temporarily retarded by the opposing force. This retardation actually starts when a slight neo-centric action takes place shortly in advance of the large one.

A similar case is represented by Fig. 14 and the graph of Fig. 15. In this case the centromere had actually reached its polar position when the neo-centric action started. The neo-centric activity of this chromosome was terminally located. The chromosome is strongly stretched during the action; and its centromere is temporarily pulled backward, *i.e.* away from the pole. After the cessation of the neo-centric action, the centromere returns to its polar position.

In both of these cases the chromosomes show an elastic re-contraction after the cessation of the pull, a re-contraction that is more rapid in Figs. 12—13 and less rapid in Figs. 14—15. The re-contraction, however, is not complete. There remains a certain lasting deformation of the chromosomes, showing that they have not only *elastic* but also *plastic* properties. They resemble in their properties both rubber and

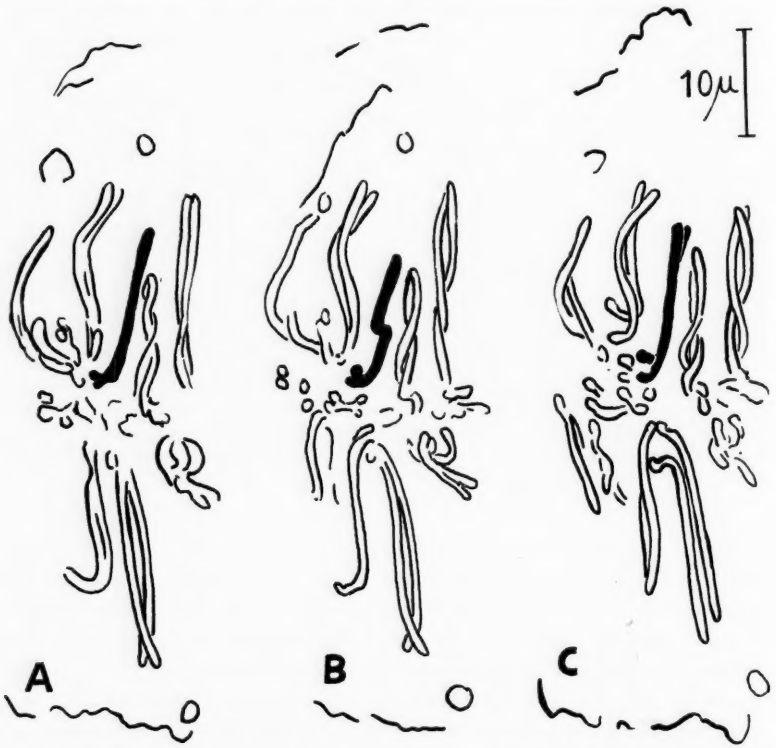


Fig. 16. A neo-centric movement in an endosperm cell of *Leucojum aestivum*. — A. Metaphase, before the movement. — B. Metaphase, during the movement. — C. Beginning of anaphase, shortly after the movement. — Only part of the mitotic figure is drawn.

chewing gum. For deformations of the size occurring in this connection we find, however, that the similarity of the chromosomes to rubber is greater than that to chewing gum.

The re-contraction of Figs. 14—15 is not only less rapid than that of Figs. 12—13 but also less complete. The difference between these two cases is easy to understand. The cell of Figs. 14—15 represents a later mitotic stage than the other cell. The chromosomes have reached the polar position and telophase is about to start. Telophase is characterized by changes in chromosome structure leading to a softening of the chromosome material. The slower and less complete re-contraction in this

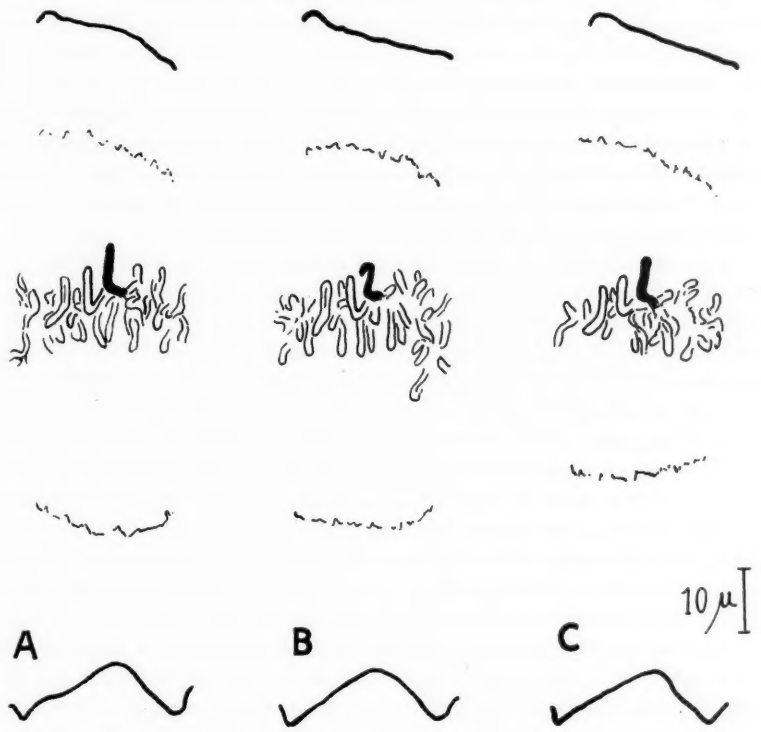


Fig. 17. A neo-centric movement in an endosperm cell at metaphase of *Clivia cyrtanthiflora*. — A. Before the movement. — B. During the movement. — C. After the movement. — Only part of the metaphase plate is drawn.

cell suggests that the first steps of this telophase softening have already started in these chromosomes. A plastic deformation of the size found in this cell has never been observed in any of the numerous cases of neo-centric movements observed in earlier mitotic stages.

It has been mentioned above that the neo-centric movements are the most rapid movements found in the chromosomes of these cells. A very instructive case is photographed in Fig. 4 D—F. In this cell a neo-centric movement occurred during anaphase in the middle region of a chromosome arm. As this neo-centric movement *took place in the same direction* as the centromere movement of this chromosome, and, as these two movements *could be studied simultaneously*, very good con-

ditions for a comparison of the two movements were found here. It can be clearly seen that the neo-centric movement is much more rapid than the centromere movement. The distance between the centromere and the neo-centric region is decreased with the result that the connecting chromosome region is bent. (The thin appearance of the most proximal region of the long arm of this chromosome is an optical artifact due to the fact that this region is slightly out of focus and its image is influenced by the phase contrast halo around the short arm.)

A surprising feature about the neo-centric action is the fact that the return movement of the neo-centric region towards its original arrangement is also often very rapid (see, *e.g.* Fig. 8, second graph from the top). The rapid return is easy to understand in cases in which the forward movement involved an elastic stretching of the chromosome, as in Figs. 12—13. However, in other instances the cause of this high speed is far from clear. If the return were merely a matter of relaxation of a bent chromosome arm, then we should expect a lower speed, something similar to the speed of straightening of the chromosome arms during prometaphase, a movement having a speed that may be as rapid as the anaphase movement but that is usually somewhat slower (MOLÈ-BAJER and ÖSTERGREN, in preparation). This question about the rapid return movements will be considered again by ÖSTERGREN (1962).

As mentioned above in the paragraph on material and methods, we have observed a neo-centric activity also in the chromosomes of the endosperm mitoses of many other species of higher plants. These were enumerated above (pp. 565—566) and their names need not be repeated here. An example from *Leucojum aestivum* is drawn in Fig. 16, and one from *Clivia cyrtanthiflora* in Fig. 17.

This type of movement was found to be present in all species investigated which had chromosomes sufficiently large to permit a study in this respect. We failed to find evidence for it only in species having chromosomes too small to permit a critical investigation of this question (such as *e.g. Colchicum*).

As was mentioned above, abrupt bending movements of the chromosomes were found also in leaf cells of the fern *Hymenophyllum tunbridgense*. In this material the cell walls prevent a successful use of phase contrast ciné-micrography, and the mitoses had to be photographed without phase contrast. For this reason it was impossible to study the abrupt bending movements in all details. It can be seen in more favourable materials, however, that such movements are caused by a neo-centric activity. For this reason we think that these movements

are evidence for the existence of a neo-centric activity also in the fern *Hymenophyllum*.

BLOOM and ZIRKLE have produced an excellent phase contrast cinémicrographic film of mitosis in the newt, *Triturus viridescens*, in which it is possible to study many aspects of chromosome behaviour in all detail. When we examined their film, we found quite clear neo-centric movements in this material also. A good example is illustrated in Fig. 18.

In the newt and related species the chromosomes show a strong tendency to be arranged on a so-called hollow spindle. This group of animals actually contribute classical material for observing this type of chromosome arrangement (*e.g.* BĚLAŘ, 1926, Fig. 15, p. 26). This means that the centromeres are usually lying in the surface layers of the spindle and the chromosome arms are hanging out laterally from the spindle in the cytoplasm. The chromosome arm showing the neo-centric activity also had this normal arrangement before the start of its neo-centric movements as well as after their cessation (Fig. 18).

In the film the arm in question can be seen to make a series of at least 3—4 neo-centric movements. The neo-centric region was terminally (or subterminally) located. During these movements the end of the arm was bent inward towards the axis of the spindle. The inward movement of the end was arrested in the surface layers of the spindle; it penetrated a short distance only into the spindle. In this respect it showed a behaviour very similar to that characteristic of the centromeres in these mitoses. Fig. 18 B shows this chromosome at the moment of maximal neo-centric activity. At this stage a small point of chromosome material protruded from the chromosome end, a deformation showing that the chromosome was subjected to a pulling force working at it.

Fig. 18 D demonstrates some of the forces that in our opinion act on centromeres in the newt type of mitosis. Similar forces most probably work also on the neo-centric chromosome end causing it to behave as shown in Fig. 18 B. These neo-centric forces are drawn in the diagram of Fig. 18 D. The behaviour of the centromeres and the neo-centric region is here in good agreement with the theoretical note of ÖSTERGREN (1945 b) on transverse equilibria in the spindle.

As the pulling forces acting on the two chromatid centromeres of a metaphase chromosome towards the two opposite poles are not directly opposing forces, their interaction gives a resultant force (marked R in the figure) working in a transverse direction inward towards the axis of the spindle. The chromosomes are prevented from moving inward,

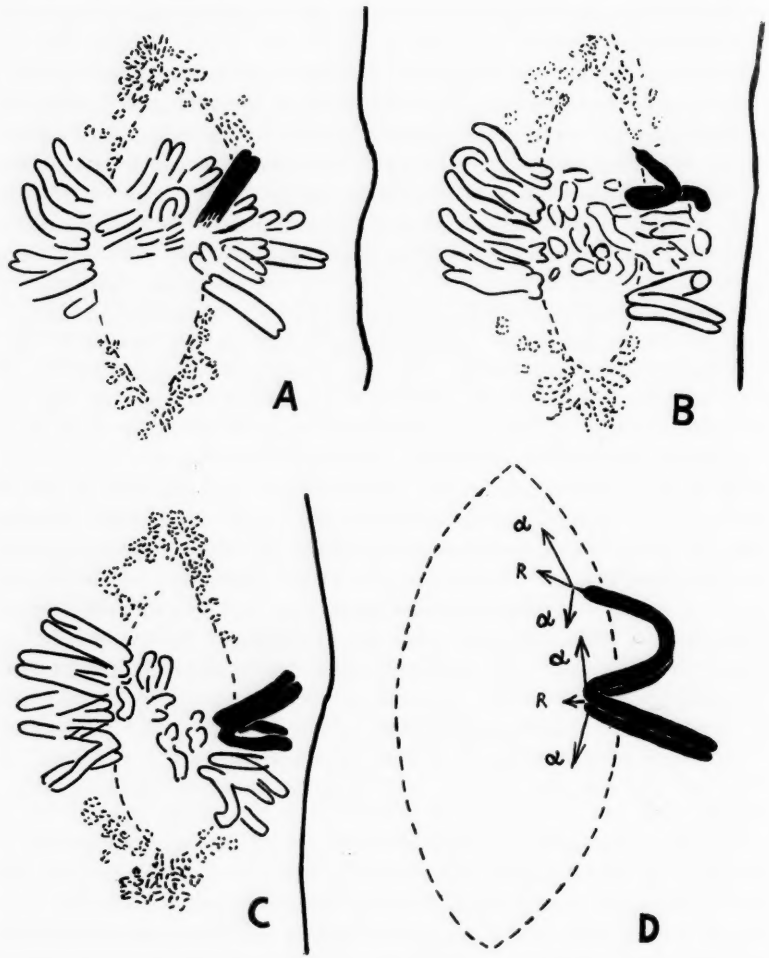


Fig. 18. Neo-centric activity of a chromosome end at metaphase in a tissue cultured cell of the newt *Triturus viridescens*. — A. Before the movement. — B. During the movement. The chromosome end is pulled inward towards the spindle axis, but the movement is stopped in the peripheral region of the spindle. (Interpretation, see Fig. D.) — C. After the movement. — D. Diagram of some of the forces influencing the position and shape of a chromosome of this kind. The traction fibres pull centromeres and neo-centric regions towards the two opposite spindle poles. The interaction of these pulling forces (marked α) gives resultant forces (marked R) directed inward towards the spindle axis. The inward movement is prevented by another factor whose

however, by other forces working in the outward direction on the chromosome arms. These forces are due to an action on the chromosome arms by the spindle that in this material strongly strives to eliminate acentric bodies out of it in the transverse direction. These outward forces on the arms have not been drawn in the figure. Thus, the position of a chromosome is here not only the result of an equilibrium between forces pulling in the two opposite poleward directions on the centromere, *but also* an equilibrium between forces working inward towards the spindle axis on the centromere and forces working outward on the chromosome arms. The existence in this material of these so-called "elimination forces" working on chromosome arms in the outward direction is well substantiated by other observations of chromosome behaviour in these mitoses (ÖSTERGREN *et al.*, 1960).

The behaviour of the neo-centric chromosome end in this cell (Fig. 18) suggests that it is attached to spindle fibres in both the opposite polar directions. These two traction fibres are of different strength and their interaction gives a resultant force acting in an oblique direction towards the spindle axis. The chromosome end moves in the direction of this force; but, when it penetrates the surface layers of the spindle, the elimination forces (by means of which the spindle system strives to remove all foreign bodies) come into action and stop the movement. For a while the chromosome end is stabilized by an equilibrium between the pulling forces acting inward and the elimination forces acting outward. After that the neo-centric action stops and the chromosome end returns to its normal cytoplasmic position.

Thus, we assumed here that the neo-centric chromosome region in the newt case was attached towards both the opposite spindle poles. As will be mentioned in the general discussion of these effects (ÖSTERGREN, 1962), it is a natural interpretation to suppose that this is the usual way of attachment of the neo-centric regions also in the endosperm mitoses.

AN ALTERNATIVE INTERPRETATION?

Pulling forces acting on chromosomes could naturally be transmitted through fibrillar structures of different kinds. A pulling influence on a chromosome region does not necessarily mean that we are concerned with the action of a chromosomal

nature is not clearly understood. This factor, called the "elimination forces" by ÖSTERGREN *et al.* (1960), acts to remove all sorts of bodies out of the organized spindle region and away from the centrosomes. The outward forces caused by the action of this factor on the chromosome arms have not been drawn in the figure. — Pictures drawn by us from the last cell of the commercially available ciné-film by BLOOM and ZIRKLE on mitosis in the newt.

spindle fibre. Thus, anaphase chromosomes in various materials are often found to be influenced by threads or strands of chromosome material that connect the separating daughter chromosomes. Such connections due to stickiness sometimes retard the chromosome movements and cause a stretching of the separating chromosomes. It has recently been suggested (JACOBSEN, 1960) that matrix connections between the chromosomes may be of some importance in influencing their distribution at various stages.

It is not our intention here to discuss the influence that matrix connections in general might have on the behaviour of the chromosomes, or the question concerning to what extent such observations on fixed material correspond to the situation in living cells. Irrespective of these problems, it might be of some interest here to consider the question whether the "neo-centric movements" studied by us might be caused by a contraction of interchromosomal connections rather than by an action of the spindle mechanism. The fact that the neo-centric activity studied by us was more pronounced in cells treated with methanol makes this problem more interesting. A treatment of this kind is capable of inducing stickiness in the chromosomes. Only slight tendencies to stickiness were observed in our material, however (ÖSTERGREN and BAJER, 1960).

We do not believe that the "neo-centric movements" studied in the present paper could be caused by interchromosomal connections. The reasons for our opinion will appear in the following discussion.

Before the neo-centric movements start, the chromosome regions in question move as freely as other chromosome regions and give no evidence of connections with other chromosomes which would hamper their movement. For example, the end of a long chromosome approaches a spindle pole in a normal way when the chromosome arms straighten towards the poles during prometaphase and early metaphase. After that, this end can be suddenly pulled towards the equator in a neo-centric movement. If this kind of behaviour is to be interpreted in terms of interchromosomal connections, then it would be necessary to assume that these connections are at first quite soft and liquid, and that they, later on, can suddenly gel, forming solid strands that perform muscle-like contractions.

A suggestion of this kind need not, in itself, be considered an impossible interpretation. When we consider its consequences for the chromosome behaviour, however, we find strong evidence against it. When a fibre causes a pull by means of a muscle-like contraction, it will induce pulling forces of the same size at both its ends. For this reason movements caused by this mechanism should occur in pairs. Each movement in a chromosome should be accompanied by a simultaneous movement in another chromosome in the opposite direction.

It is true, that in many cases one of the movements might be concealed by the chromosome in question being out of focus, or because other chromosomes might conceal it. In many flattened cells, however, neo-centric movements could be observed by us under such conditions that a corresponding opposite movement in another chromosome could not possibly have been concealed. In this way it could be ascertained that the causal agent of many of these movements could not be a contractile interchromosomal connection. This is, of course, very clear also for the movements (*e.g.* Fig. 6) which are performed in the direction of the nearest spindle pole. In such cases there is usually no other chromosome present in the direction of the movement.

In numerous cells, especially in untreated material, the long chromosome arms are arranged more or less parallel to one another in the direction from the equator towards the spindle poles. Interchromosomal connections arranged at random should frequently lie across the spindle between such parallel chromosome arms. If they were to contract we ought to see pairwise movements across the spindle in which certain regions of these parallel arms approach one another. No movements of this kind were found, however.

As our neo-centric movements usually are directed towards the equator; and, as they are not too rare, we should naturally sometimes find the situation that chromosomes lying on opposite sides of the equator perform movements simultaneously towards the equator, movements that may even give the impression that the neocentric regions move towards one another. Two or three instances of this behaviour were observed.

Many of the movements that we are concerned with in the present paper are obviously produced by a mechanism which does not work through interchromosomal connections. It is naturally conceivable that a few movements of this kind might be caused by another mechanism that depends on such connections. In our opinion, however, there is at present no evidence that justifies an assumption of this kind.

The neo-centric movements are not found in endosperm cells in which the spindle has been disorganized by colchicine treatment (MOLÈ-BAJER, 1958; BAJER, 1958 b). This fact, of course, suggests that these movements are caused by the spindle mechanism. This opinion is supported by the direction of the movements in our study usually being along the fibre structure of the spindle. The neo-centric regions of the chromosomes were seen to be attached to chromosomal spindle fibres in the studies by ÖSTERGREN and PRAKKEN (1946) and RHOADES (1952) on fixed material of meiosis in rye and maize.

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SUMMARY

During their ciné-micrographic studies on mitosis in the endosperm BAJER and MOLÈ-BAJER (1956) and especially BAJER (1958 b) found that the chromosome arms often made sudden bends in the direction towards the spindle equator. ÖSTERGREN and BAJER (1960) found that these "abrupt bending movements" were caused by the action of pulling forces on small regions in the chromosome arms. These forces often stretch the regions in question to pointed tips. According to the interpretation given in that paper these pulling forces result from the appearance of short-lasting periods of neo-centric activity in these small chromosome regions. This interpretation means that these regions would temporarily organize chromosomal spindle fibres of the same kind as found at the centromeres. An effect of this kind is earlier known from studies on fixed material of meiosis in various plant species. The expression "neo-centric activity" was introduced by RHOADES (1952). In these meiotic cases the effect must usually be of longer duration, however.

In the present paper this transient neo-centric activity of mitotic chromosomes is investigated in more detail. As in our previous studies ciné-micrography of living cells on 16 mm film was used. The phenomenon is most easily studied in endosperm mitoses of species with large chromosomes. For this reason most of the work was done on *Haemanthus katherinae*. The effect is found, however, also in the endosperm mitosis of many other species. Abrupt bending movements of the chromosome arms, most probably caused by the same mechanism, were seen in leaf cells of the fern *Hymenophyllum tunbridgense*. A transient neo-centric activity was observed by us also in the film by BLOOM and ZIRKLE on mitosis in the newt.

At metaphase in the endosperm mitoses, the chromosome arms are usually arranged from the equatorially located centromeres in the directions of the two opposite spindle poles. This arrangement is temporarily disturbed by the neo-centric activity which causes short-lasting very rapid movements of small strictly localized chromosome regions, usually in the direction towards the spindle equator. These movements are the most rapid of all the chromosomal movements. In *Haemanthus* they often reach the order of 5 μ/min, while the maximum speed of the anaphase movements is less than 2 μ/min. Usually the neo-centric movements last for a few minutes only in each instance, a fact that prevents them from interfering with the normal distribution of the chromosomes

to the daughter nuclei. The duration of the prometaphase stage is of the order of 110 min and that of the metaphase 45 min. The deformation induced in the chromosomes by the neo-centric activity demonstrates that the movements are caused by pulling forces. Besides the equatorially directed movements there are also movements towards the nearest spindle pole and movements in a direction transverse or oblique to the spindle structure (Table 1).

These neo-centric movements are performed by the chromosomes during the prometaphase, metaphase and anaphase stages. The neo-centric regions can appear in any position along the chromosome arms from the ends to positions close to the centromeres.

In all instances permitting a critical test of this question, the neo-centric activity at metaphase appeared in only one of the two chromatids at each movement. When repeated movements were performed, however, the activity could change from one chromatid to its sister, the pull influencing now the one now the other of the two chromatids. In one case the evidence even suggested that only a single half-chromatid of a metaphase chromosome was influenced.

In cells treated with methanol the neo-centric movements appeared with an increased frequency and with stronger deformation of the chromosomes.

Observations on neo-centric activity in acentric fragments and non-chromosomal bodies are considered in the report of ÖSTERGREN and BAJER (1962), and a discussion of certain theoretical aspects of these effects with a review of related observations from the literature is given by ÖSTERGREN (1962).

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FREQUENCIES OF HAPTOGLOBIN TYPES IN THE SWEDISH POPULATION

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I. INTRODUCTION

BY a special method of starch gel electrophoresis, introduced by SMITHIES (1955 a and b), human sera can be shown to contain distinct protein components, after staining visualized as a structural pattern. In this starch gel medium, the migration of some molecules seem to depend not only on their electrical charge but also on their molecular size which gives a high degree of resolution. SMITHIES found three different patterns of haemoglobin-binding proteins (cf. Fig. 1), which later were identified as the haptoglobins described by JAYLE (1939). SMITHIES and WALKER (1955) analysed the distributions in 18 families and concluded that these three serum types were genetically determined by free combination of two autosomal genes without dominance. Their results have been confirmed in further family investigations by FLEISCHER and LUNDEVALL (1957), GALATIUS-JENSEN (1958 a), HARRIS, ROBSON and SINISCALCO (1958) and MÄKELÄ, ERIKSSON and LEHTOVAARA (1959). However, some cases of atypical segregation were reported by HARRIS and co-workers. SMITHIES and WALKER (1956) suggested the notation used in this paper. At first, the three groups were called I, II A and II B.

ALLISON, BLUMBERG and AP REES (1958) reported a fairly high frequency of individuals without visible haptoglobin pattern in Nigerian Africans. SUTTON, NEEL, BINSON and ZUELZER (1956) and GIBLETT (1959) found a high frequency of the Hp^1 gene in Negroes. Low frequencies of this gene have among others been found in Alaskan Eskimos (BLUMBERG, ALLISON and GARRY, 1959) and in Swedish Lapps (BECKMAN and MELLBIN, 1959).

In addition to the three normal haptoglobin patterns, some modified types have been reported. GIBLETT (1959 a) found about 10 per cent of a modified Hp 2—1 type in American Negroes from Seattle. This type is

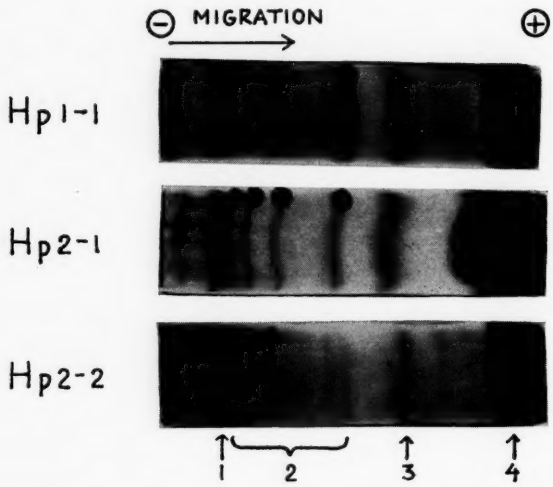

Fig. 1. Photographs of starch gels showing the three different common haptoglobin types. The haptoglobin components are indicated by black dots. 1, slow a_2-globulin; 2, haemoglobin-haptoglobin-complex; 3, transferrin and 4, albumin.

TABLE 1. *Frequencies of haptoglobin types in Swedish Lapps and Swedes from Central Sweden.*

Population	Number tested	Haptoglobin types								Authors
		1–1		2–1		2–2		No haptoglobin		
		n	%	n	%	n	%	n	%	
Swedes (central Sweden)	1003	153	15.2	455	44.4	402	40.1	3	0.3	TARUKOSKI, 1959
Lapps (county of Norrbotten)										
Karesuando										
Lannavaara	112	6	5.4	49	43.8	54	48.2	3	2.7	BECKMAN and MELLBIN, 1959
Jukkasjärvi	68	6	8.8	28	41.2	32	47.1	2	2.9	—„—
Gällivare	47	5	10.6	20	42.6	21	44.7	1	2.1	—„—
Jokkmokk	67	9	13.4	32	47.8	26	38.8	0	0.0	—„—
Arjeplog	35	5	14.3	13	37.1	16	45.7	0	0.0	—„—
Lapps (county of Jämtland)	40	1	2.5	13	32.5	26	65.0	0	0.0	BECKMAN, unpubl. data

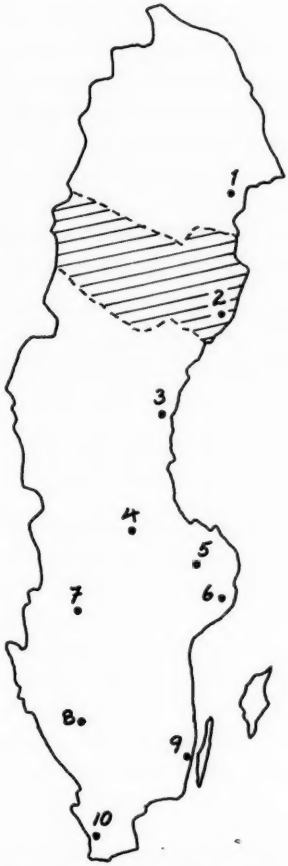

Fig. 2. Map showing the locations of the different samples.
Hatched area: the county of Västerbotten.

characterized by a higher concentration of haemoglobin-haptoglobin in the fast-migrating bands. GALATIUS-JENSEN (1958 b) described a rare type called Hp Ca in the Danish population. BLUMBERG, ALLISON and GARRY (1959) described two other modified types, and GIBLETT (1959 b) observed a rare haptoglobin type in an American Negro female and her daughter. It appears that all the types deviating from the three common patterns are modified heterozygotes.

TARUKOSKI (1959) examined about 1,000 individuals from Central

Sweden, and BECKMAN and MELLBIN (1959) studied a sample of 329 Swedish Lapps. These figures are given in Table 1, together with data from a small sample of south Swedish Lapps. The Lapps have a lower frequency of the Hp^1 gene, which can be traced also in the small south Lappish series.

II. RESULTS OF THE INVESTIGATION

This investigation includes a study of haptoglobin frequencies in samples from different parts of Sweden and an analysis of a mother—child material obtained from paternity investigations performed at the State Institute for Blood Group Serology, Stockholm.

TABLE 2. *Frequencies of haptoglobin types in samples from different parts of Sweden.*

		Haptoglobin types			No. tested	χ^2	Genes %	
		1—1	2—1	2—2			Hp^1	Hp^2
1. Boden	obs.	10	57	37	104	3.18	37.0	63.0
	exp.	14.1	48.5	41.3				
2. Umeå	obs.	13	36	39	88	0.93	35.2	64.8
	exp.	10.9	40.1	37.0				
3. Sundsvall	obs.	16	40	42	98	1.43	36.7	63.3
	exp.	13.2	45.5	39.3				
4. Falun	obs.	31	104	62	197	1.36	42.1	57.9
	exp.	34.9	96.0	66.1				
5. Uppsala	obs.	45	123	94	262	0.19	40.6	59.4
	exp.	43.2	126.4	92.4				
6. Stockholm	obs.	15	46	36	97	0.002	39.2	60.8
	exp.	14.9	46.2	35.9				
7. Karlstad	obs.	15	53	26	94	1.96	44.1	55.9
	exp.	18.3	46.3	29.4				
8. Borås	obs.	22	62	50	134	0.14	39.6	60.4
	exp.	21	64.1	48.9				
9. Kalmar	obs.	14	49	35	98	0.22	39.3	60.7
	exp.	15.1	46.8	36.1				
10. Malmö	obs.	12	45	43	100	0.002	34.5	65.5
	exp.	11.9	45.2	42.9				
Total	obs.	193	615	464	1272	0.21	39.3	60.7
	exp.	196.4	606.9	468.7				

1. Frequency data

Samples were collected from the blood donor centres of nine different Swedish towns. One sample (Stockholm) consists of individuals examined in paternity cases. The locations of the samples are shown in Fig. 2. The numerals of the map refer to the figures in Table 2.

The classification generally caused no problem. In seven cases no haptoglobin pattern could be detected. The agreement between observed and expected frequencies is satisfactory for all the samples. The regional variations of the gene frequencies are not especially marked ($\chi^2=7.64$; 9 d.f.; $0.7>P>0.5$).

As it is known that Asiatic populations (KIRK, LAI, MAHMOOD and BHAGWAN SINGH, 1960) have low Hp^1 frequencies, one would expect to find a decline of decreasing frequencies of the Hp^1 gene eastwards. The Lapps, which are sometimes believed to contain Asiatic ethnical elements, also show rather low Hp^1 frequencies. The three northernmost samples show slightly decreased Hp^1 frequencies, but the difference is not significant.

Table 3 shows the frequencies of haptoglobin types in mothers and children tested in paternity cases and in a sample of conscripts from the county of Västerbotten. The mother—child material is collected from the whole country. The location of the county of Västerbotten is indicated on the map. Also in these samples, there is a good agreement between observed and expected numbers. In agreement with the other samples from North Sweden the frequency of the Hp^1 gene is relatively low in the conscript series. The gene frequencies in the mother and child samples show rather close agreement with those of the total material in Table 2.

TABLE 3. *Frequencies of haptoglobin types in samples of mothers and children tested in paternity cases and in conscripts from the county of Västerbotten.*

Sample		Haptoglobin types			No. tested	χ^2	Genes %	
		1–1	2–1	2–2			Hp^1	Hp^2
Conscripts, Västerbotten	obs.	80	255	228	563	0.41	36.9	63.1
	exp.	76.6	262.2	224.2				
Mothers	obs.	91	254	219	564	1.43	38.7	61.3
	exp.	84.5	267.6	211.9				
Children	obs.	75	268	221	564	0.20	37.1	62.9
	exp.	77.6	263.2	223.2				

2. Mother—child combinations and the application of haptoglobin groups in forensic medicine

Next to complete family data from families checked for legitimacy of the children, mother—child combinations represent the best material for controlling the inheritance of a given trait. All family and mother—child data except the cases of atypical segregation reported by HARRIS and co-workers have so far been compatible with the hypothesis of inheritance put forward by SMITHIES and FORD-WALKER (1955).

In the mother—child material investigated there are no exceptions from the two-allele hypothesis of inheritance, and the agreements between observed and expected numbers are good (cf. Table 4). The gene

TABLE 4. *Mother—child combinations of haptoglobin groups in a mother—child material examined in paternity cases.*

Mother		Child			Total
		Hp 1—1	Hp 2—1	Hp 2—2	
Hp 1—1	obs.	35	56	0	91
	exp.	35.2	55.8	0	
Hp 2—1	obs.	40	129	85	254
	exp.	49.1	127	77.9	
Hp 2—2	obs.	0	83	136	219
	exp.	0	84.8	134.2	
Total		75	268	221	564

frequencies in the mother-series has been used for the calculation of the expected figures. When the mother is Hp 2—1 there is a slight deficiency of Hp 1—1 and an excess of Hp 2—2 children, but these differences are not significant.

The present observations further support the previous informations concerning the validity of the two-allele hypothesis and suggest that the haptoglobin system is of definite value in forensic medicine. The haptoglobin system gives a high theoretical probability of "excluding" the paternity of wrongly accused men. For a Hp^1 gene frequency of 0.39 this theoretical probability is 0.181. Using the A_1A_2B0-, MN-, P- and Rh (CcDE) blood groups and the gene frequencies occurring in the Swedish population one will get a compound theoretical probability of 0.515. By the addition of the haptoglobin system the compound probability will be increased to 0.608.

SUMMARY

The frequencies of the haptoglobin types have been studied in 1,835 unrelated adult Swedish individuals (blood donors and conscripts) and in 564 mother—child pairs. The geographical variations of the gene frequencies are generally slight. Possibly there may be lower frequencies of the Hp^1 gene in North Sweden. The mother—child combinations show a good agreement with the existing two-allele hypothesis of inheritance.

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INDUCTION OF SOMATIC CHANGES IN *SOLANUM TUBEROSUM* BY ACUTE GAMMA IRRADIATION

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IN addition to the conventional potato-breeding methods based on sexual principles, research on the development of new methods has been conducted on four lines: (1) interspecific and intervarietal grafting, (2) colchicine treatment, (3) clonal selection, and (4) ionizing irradiation. So far, however, only the two last-mentioned methods have yielded results indicating a possibility of practical utilization.

The first attempts to induce somatic changes in the potato by means of ionizing radiation were carried out by ASSEYEVA and BLAGOVIDOVA (1935), who demonstrated that X-radiation could cause permanent, vegetative alterations leading to a chimaerical structure of the plant. However, it was not until the experiments with radiophosphorus by STANTON and SINCLAIR (1951) and with X-rays and radiophosphorus by HAGBERG and NYBOM (1954) that research on this field was taken up again.

In a recent work, HEIKEN (1960) investigated the somatic effects of X-radiation on five varieties of cultivated potato. In three experimental series, the first of which was commenced in 1954, 3,420 tuber-halves were treated with doses ranging from 250 to 8,000 r. In this material 109 somatic aberrations were obtained. The highest aberration frequency amounted to 17.5 per cent and was obtained in the variety President. Parallel with the final experiment of the second series, a preparatory γ-ray experiment was carried out. The results of this experiment will be presented and discussed below.

MATERIAL AND METHODS

From the 1956 harvest, 120 tubers of the variety President were selected for evenness of size (40—45 mm). They originated from the cultivations of virus X-tested seed potatoes situated in the neighbourhood

of Umeå, about 64° N, belonging to the Institute for Plant Research and Cold Storage. This area is particularly suited for growing high-grade seed potatoes because of the almost total absence of the insect vectors necessary for the transmission of the viruses A, Y, and leaf roll. The tubers were distributed in wooden boxes and put into store in a ventilated cold-store room in the refrigeration department of the Institute. The temperature was automatically regulated to $4.5 \pm 0.5°$ C. The relative humidity of the air ranged between 85 and 90 per cent. Before the treatment on May 10th 1957, the tubers were divided into six equal groups and halved longitudinally. One half of each tuber was γ-irradiated, the other served as control.

By kind co-operation of Dr. MAIRE JAARMA, the γ-rays were obtained acute from the outdoor Co^{60} source of the Department of Genetics, Forest Research Institute, Stockholm (Head: Professor ÅKE GUSTAFSSON). A Victoreen-type air-walled ionizing chamber was used to determine the intensity of the aggregate; it was found to be 85.75 r/hour at a distance of 1 m. The samples were irradiated at three distances and the doses calculated according to the square law. The doses 250 and 500 r were given at 57 cm, 1,000 r at 28 cm, and 2,000, 4,000, 8,000 r at 20 cm. At the time of treatment, the water content of the tubers was found to be 79.23 per cent.

In the lay-out on June 5th, the tuber-halves were spaced, the control half exactly opposite the irradiated one, in two rows 70 cm apart with 35 cm between the plants in each row to make the morphological investigation as reliable as possible. The vegetative generations of tuber-halves treated with γ-rays will, in the following, be denoted by $v\gamma_1$, $v\gamma_2$, *etc.*

EFFECTS OF RADIATION IN $v\gamma_1$ AND $v\gamma_2$

Although the γ-rays from Co^{60} have a relatively low ionizing density, they have been reported to be closely related to X-rays in their general biological effects (*cf.*, *e.g.* GRAY, 1952; EHRENBERG, 1954; EHRENBERG and NYBOM, 1954). In the present study, the influence of γ-radiation on lethality, tuberization, and yield can be seen from Table 1. As is evident, there was a suppressing effect of radiation increasing from the lowest to the highest doses. Failing ability to reproduce vegetatively was chosen as the criterion of lethality. Owing to diseases, some plants were removed from the experimental field during the period of growth. At the highest doses many tuber-halves were so heavily affected that they

TABLE 1. *Lethality, tuberization, and yield in* $v\gamma_1$.

Date of treatment, planting and harvest	Variety	Dose, r	Lethality, %		No. of plants harvested	Mean number of tubers per plant			Mean weight of tubers per plant, g		
			Control	Treated		Control	Treated	Diff.	Control	Treated	Diff.
May 10th June 4th September 17th	President	250	0	0	19	21.2±1.42	16.2±1.25	4.7[1]	905±60.8	694±55.7	211[1]
		500	0	0	20	25.9±2.07	18.0±1.17	7.9[2]	1072±69.3	767±60.0	305[2]
		1000	0	0	18	23.1±1.93	14.7±1.85	8.4[2]	822±52.0	527±66.3	295[2]
		2000	0	0	18	23.1±1.64	15.2±1.18	7.9[3]	904±54.8	616±37.4	288[3]
		4000	0	10	18	18.7±1.70	8.3±0.90	10.4[3]	707±80.0	408±60.8	299[2]
		8000	0	45	11	18.4±2.05	6.2±0.94	12.2[3]	1075±93.3	138±28.3	937[3]

[1] $P<0.05$; [2] $P<0.01$; [3] $P<0.001$.

remained ungerminated. When lifted, such tuber-halves were usually intact and showed no shrinkage or softening.

The threshold value, giving about 50 per cent lethality, was found to be 8,000 r as in the parallel X-ray experiment. However, it is known from the other experimental series that the radiation sensitivity of the potato is strongly influenced by environmental conditions during the period of growth and by the physiological stage of the tubers at the time of treatment. Further, different varieties may show significant differences in their reactions. Mainly because of these factors, the critical dose may range between 4,000 and 8,000 r. ASSEYEVA and BLAGOVIDOVA (*l.c.*) observed that most artificially produced somatic aberrations in *Solanum tuberosum* are analogous with the spontaneous ones described in the literature. This observation was confirmed in the previous and the present investigation.

In $v\gamma_1$ no permanent deviations were detected. In $v\gamma_2$, the following aberrations were obtained and found to be permanent during three year's propagation:

Light-green-leaf (500 r). — Spontaneous forms of this aberration have been observed by FRUWIRTH (1928) and HEIKEN (*l.c.*), who proved the existence of a periclinal structure and a slower sprouting of the tubers. The aberration has also been induced by X-irradiation (HEIKEN, *l.c.*).

Leaf deformities (1,000 r). — Several more or less marked spontaneous leaf-deformity types, rather unstable, but of a permanent nature, have been isolated by ASSEYEVA (1927, 1931) and HEIKEN (*l.c.*), who also induced the type by X-irradiation. The type found during the present investigation exhibited, besides leaf-deformities, coalescent leaflets.

Dwarf (1,000 and 8,000 r). — As far as is known, the only spontaneous dwarf type described in the literature has been observed by RIEMAN *et al.* (1951). However, dwarfs, constituting a group of poor types ranging from such as deviate from the original varieties mainly by somewhat reduced growth to very drastically diminished forms, have been induced by X-irradiation (HEIKEN, *l.c.*). The two forms isolated during the present γ-ray experiment, showed a pronounced dwarfism including almost all organs. In addition, the leaves were coarse.

Increased pigmentation of corolla (2,000 r). — Loss aberrations, changing the flower colour from blue-purple or red-purple to white, have been frequently recorded in the literature. Spontaneous gain aberrations affecting the colour of the flower are, on the contrary, extraordinarily rare, and they have not previously been induced by ionizing

radiation. In fact only two cases, one of which is somewhat obscure, are known to have been reported. Thus, applying an eye-excision technique, ASSEYEVA (1927) demonstrated that the variety Tsungunka, characterized by blue-purple flowers, was an aberrant form of the variety l'Institut de Beauvais, characterized by white flowers. Furthermore, a type with changed (apparently increased) colour of the corolla has been observed in the variety Pac and described by KOĆNAR and SMERDA (1928). However, this aberration was detected by the comparison of two commonly cultivated stocks, and it was not possible to trace back the change to its origin with certainty. The γ-ray-induced type isolated during the present experiment exhibited a dark purple colour of the corolla rather similar to the colour characteristic of the variety Konsuragis.

Depigmentation of corolla (4,000 r). — As mentioned above, depigmentation of the flower has been observed in several varieties. In the variety President, depigmentation has been reported by SALAMAN (1926), MCINTOSH (1945), and WHITEHEAD *et al.* (1953). A white colour of the flower has also been induced by X-irradiation in this variety (HEIKEN, *l.c.*). Unfortunately, the white flowering plant isolated in the present study was found to be virus X infected, but it seems hardly possible that the virus infection had any influence on the change of colour (*cf.* the grafting test mentioned below). In earlier experiments, HEIKEN (*l.c.*) has demonstrated the chimaerical structure of this type.

Small-leaflet (8,000 r). — Spontaneous small-leaflet forms have been observed by RIEMAN *et al.* (*l.c.*) and induced by X-irradiation by HEIKEN (*l.c.*). The γ-ray-induced small-leaflet type isolated in this experiment was characterized, in addition to the diminution of the leaflets, by a duller green colour than the control plants.

All aberrant types induced were checked for the presence of the virusus X and S by a serological method (*cf.* SLOGTEREN, 1955), and for the presence of the viruses A and Y by inocculation in *Solanum demissum*. At the start of the experiment, seed material free from virus S was not available in Sweden, and a laboratory examination revealed that all the plants were infected with this virus. As for the other viruses, the only case to occur was that mentioned above. However, the inability of the isolated plants to transmit their characteristic features was proved by grafting. On the basis of this test it may be concluded that the aberrations obtained were not due to virus infection.

As is seen, 7 aberrations occurred in the γ-irradiated material (*i.e.* 6.7 per cent). The control plants were examined just as carefully as

the treated material, and it may safely be maintained that observations of the types recorded in the irradiated material would have been recorded to the same extent in the control material. However, no aberrations occurred in the control.

In the parallel X-ray experiment (in which the material was stored, planted, lifted, *etc.*, in exactly the same way as that described above), the number of aberrations amounted to 15 (*i.e.* 14.3 per cent). An analysis of the results by calculating heterogeneity — χ^2 shows that the difference is not statistically significant ($\chi^2=3.15$; d.f.$=1$; $0.10>P>0.05$). Therefore, until further investigations have been carried out, both mutagens seem equally valuable in practical potato-breeding when the γ-dosage is given acute.

DISCUSSION

Because of the vegetative reproduction of the potato, mutations with a slight effect on physiological or biochemical properties may be directly utilized in agriculture. As a matter of fact, a considerable number of valuable commercial varieties are known to have originated by spontaneous mutation. In 1951, KRANTZ reported that varieties, the origin of which could be traced back to somatic mutations in cultivated potato clones, represented about 15 per cent of the total certified seed production in the U.S.A. According to a recent report by TURNQUIST (1960), they amount at present to about 35 per cent. In Germany, BERGANN (1955, 1957) has adopted an optimistic attitude towards the practical utilization of the spontaneous variability of the potato and in other parts of the world interesting and promising clonal selection experiments have been carried out (*cf.*, *e.g.* BALD and OLDAKER, 1950; BRUST, 1951; RIEMAN *et al.*, 1951; DAVIDSON and LAWLEY, 1953; COCKERHAM and MCARTHUR, 1956).

In plant-breeding concerned with self-fertilizing species, the basic purpose of inducing mutations has been to accumulate new raw material for continued recombination by hybridization (GUSTAFSSON, 1947; GUSTAFSSON and TEDIN, 1954; GUSTAFSSON *et al.*, 1960). Since it is now possible, by the aid of ionizing radiation, to transform the anatomical structure of periclinal chimaeras in such a way that a mutation in the epidermis may be made to include the sexual tissue as well (ASSEYEVA, 1931; HOWARD, 1958), new and favourable induced characteristics may be introduced in potato-breeding in a similar way.

This preparatory experiment and the previous experiments in the

series are, of course, insufficient to provide a sure basis for predictions regarding the value of γ- and X-radiation in potato-breeding, but the aberration frequencies obtained have been high enough to make continued research on this field worth-while. The greatest difficulties are those which also occur with spontaneous aberrations, *viz.* the striking pleiotropic effect of most somatic changes and, technically, the necessity of finding and maintaining virus-free material in order (1) to be able to recognize minor aberrations, (2) to ensure that minor aberrations observed do not depend upon mutations induced in the viruses themselves, and (3) to bring the method parallel with the production of new clones from true seeds.

As pointed out by many mutation workers, most induced mutants may be expected to be poor forms, which are worthless from an economic point of view; from time to time, however, types may occur which go beyond the limits of natural variation. In the present study, the dark purple flowering type induced possess a special interest by apparently representing a slight gain mutation extraordinarily seldom produced by nature itself.

SUMMARY

(1) The purpose of the present study was to gain a preliminary insight into the mutagenic somatic effects of acute γ-radiation on the potato. The experiment was commenced in 1957 and included the variety President.

(2) In the first vegetative generation, there was a clear suppressing effect of radiation increasing from the lowest to the highest doses (250—8,000 r). The threshold value, giving about 50 per cent lethality, was found to be 8,000 r. However, on the basis of previous X-ray experiments, this value was regarded as an upper limit only reached under favourable conditions.

(3) In the second vegetative generation, seven somatic aberrations were isolated. In relation to the total number of surviving families, they amounted to 6.7 per cent. The permanence of the types induced was proved during three years' propagation.

(4) Due to the fact that changes in the morphology of the potato may be caused by virus infection, it was found necessary to check all the types induced. Of the tests for the presence of viruses, that of transmission by grafting was considered the most conclusive. However, none of the aberrant forms isolated transmitted their characteristic features.

(5) Finally, the experimental results were discussed from a breeding point of view.

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STUDIES OF THE CHROMOSOMES IN AMERICAN OBESE-HYPERGLYCAEMIC MICE

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INTRODUCTION

AMERICAN obese-hyperglycaemic mice were first discovered as a new mutant in 1949 (INGALLS, DICKIE and SNELL, 1950). They increase rapidly in weight until they are about three times the weight of normal animals. The affected animals are usually sterile, probably due to the obesity, but when kept on a restricted diet some obese males have produced offspring (LANE and DICKIE, 1954). Offspring have also been obtained from sterile obese female mice following hormonally induced ovulation and transplantation of resulting eggs to fertile recipients (RUNNER and GATES, 1954). All these investigations show that the obese animals are homozygous for a recessive gene (*ob*).

The chromosomes of obese mice have not previously been studied. In spite of the small chance of finding numerical or morphological chromosomal aberrations in this condition, it was considered worthwhile to study the chromosomes using the new simple technique described below.

MATERIAL AND METHODS

The chromosomes were studied in four mice of the strain with the recessive trait of diabetes obesitas, which originated through a mutation at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, USA. Two obese-hyperglycaemic animals (AO mice), one of each sex, 2 months and 4 months old were examined. Two lean litter mates (AN mice) of each sex were used as controls (see Table 1).

The technique used is a slight modification of that described by FECHHEIMER (1960), in which chromosome preparations are made from the epithelial layer of the cornea.

The animals were injected intraperitoneally with 0.01 ml of 0.04 per

TABLE 1. *Number of scored cells and their chromosome number from different mice.*

AO=American Obese-hyperglycaemic mice, AN=Lean control mice.

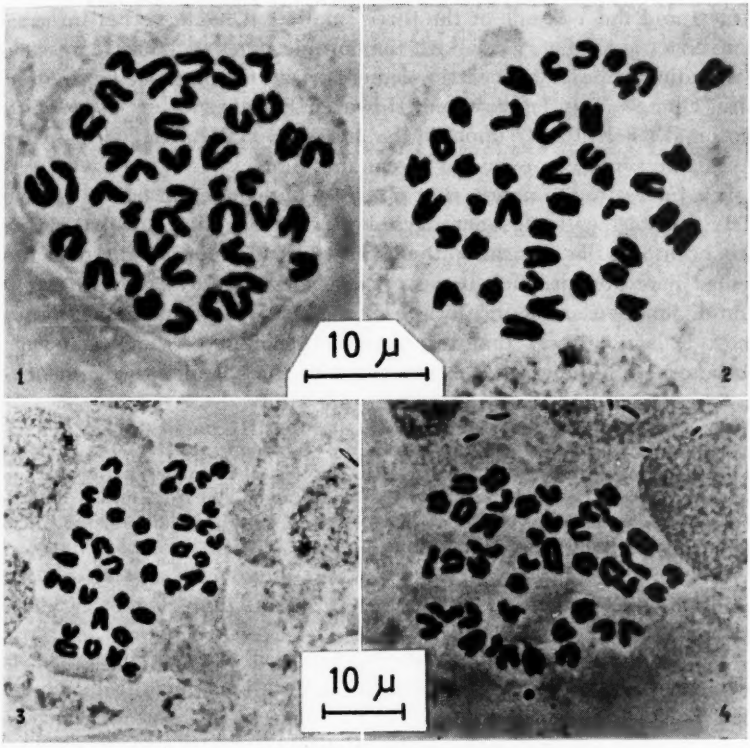
	Age months	Weight gr.	Chromosome number						Cells counted	
			39 ?	39	40 ?	40	41 ?	41	Certain	Total
AO ♂	4	50		1	1	24			25	26
AN ♂	4	35		1	1	23		1	25	26
AO ♀	2	35	1		2	24		1	25	28
AN ♀	2	15	1	1	1	24	1		25	28
			2	3	5	95	1	2	100	108

cent aqueous colchicine solution/gm. of body weight. After 3½ hrs the animals were killed, the eyes cut out and immediately placed in hypotonic solution (0.3 per cent sodium chloride at 37° C) for 45 min. The complete eye was then fixed for 10 min. in 45 per cent acetic acid and stained 2 min. in orcein [a solution of 2 per cent natural orcein (T. G. Gurr) in 50 per cent acetic acid]. Pieces of the epithelial layer were scraped from the cornea into a drop of orcein on a slide (not albuminized). A coverslip was placed over the drop, and the tissue was first carefully squashed by manual pressure, then finally in a press. The preparation was then sealed with a mixture of 50 per cent collophonium and 50 per cent paraffin.

Preparations made in this simple way offer good possibilities for chromosomal analysis, but these preparations are not of a permanent nature. More than 1,300 mitoses were counted in one preparation made in this way from a single eye and usually it is possible to determine unequivocally the chromosome number in 30—40 selected metaphases.

RESULTS AND DISCUSSION

Apparently suitable metaphases were chosen using relatively low magnification (160 ×). The chromosomes were then drawn and counted under high magnification (1,000 ×). In the majority of the cells chosen in this way it was possible to make reliable determinations of the chromosome number. This could be done because: 1) the chromosomes were intensely stained and distinct from one another, 2) all chromosomes were in one plane and were well concentrated in a metaphase plate and 3) no single, detached chromosomes were found outside the plates. Of



Figs. 1—2, corneal cells showing the normal chromosome number of 40. Fig. 1 from a control male mouse, Fig. 2 from an obese-hyperglycaemic female mouse. — Figs. 3—4, examples of cells in which mitotic non-disjunction has occurred. Both from control mice, Fig. 3 from a female showing 39 chromosomes, Fig. 4 from a male showing 41 chromosomes.

108 cells counted, 100 were considered as reliable with respect to these conditions (see Table 1). Of these 100 cells, 95 had the normal chromosome number of 40 (Figs. 1, 2), 3 cells 39 and 2 cells 41 (Figs. 3, 4). From each mouse 25 clear cells were analysed. There was no difference in the number of chromosomes between the AO and AN mice. Further, it was not possible to find any aberration in the morphology of the chromosomes from the AO mice.

It is impossible with all techniques used so far to identify the individual chromosomes of the mouse [the X-chromosome is one of the

largest and the Y is one of the three smallest (LEVAN, verbal information; STICH and HSU, 1960)]. All the chromosomes together represent a descending scale of telocentric elements. Due to this only large structural changes may be observed. However, the absence of visible chromosomal aberrations supports the hypothesis that the obese-hyperglycaemic syndrome is due to a gene mutation.

It is interesting to notice, that mitotic non-disjunction occurs in vivo. It is possible, with this new technique, to determine to what degree this occurs in the corneal epithelial layer of the mouse. The data presented here are insufficient, but, nevertheless, indicate that effects of non-disjunction are found in approximately 5 per cent of the corneal cells. It is not likely that the observed deviations from the normal chromosome number can be attributed to the colchicine treatment or to other technical procedures used in this study.

Acknowledgements. — I wish to express my gratitude to BO HELLMAN, M.D. and to CLAES HELLERSTRÖM, M.D., both of the Histological Department, University of Uppsala, Sweden. They first suggested this investigation, and from them I obtained the experimental animals. This work was aided by a grant from the Swedish Atomic Research Council to Professor J. A. BÖÖK.

SUMMARY

The chromosomes of American obese-hyperglycaemic mice (AO) and lean litter mates (AN) were studied in preparations made from the cornea. The technique is described. No differences with respect to chromosome number and morphology have been observed between the AO and the AN mice. Aberrations from the normal chromosome number due to mitotic non-disjunction were found in both AO and AN mice.

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DIVERSITY OF INTERACTION PROPERTIES IN EIGHT BACK-MUTATIONS FROM THE MUTANT w^a IN DROSOPHILA MELANOGATER

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IN a previous paper (RASMUSON, GREEN and EWERTSON, 1960) eight reverse mutations from the *apricot* mutant of the *white* locus of *Drosophila melanogaster* were analyzed for their content of red and brown eye pigments and pteridines. It was found that none of the reversions had accomplished a complete restoration of the wild type gene function. It was also found that some of the reversions agreed very closely as to their amounts of pigments and pteridines. Whether this agreement was due to a real mutational identity or not could not be decided without further tests involving interactions with other genes. A decision in this matter would however be of great significance for the apprehension of the number of reversion possibilities and for the understanding of the gene function. Therefore, in the present investigation the same w^a reversions have been studied in combination with four genes, all known to interact with the expression of the apricot character. Only the total amounts of red and brown pigments have been studied, since it was found in the previous study that these characteristics were the most informative in distinguishing the genotypes.

MATERIAL AND METHODS

The origin of the eight reversions has been accounted for in the previous paper. Here they will simply be referred to as R1 to R8. The four genes with which they have been combined are:

1. suppressor of w^a, *su-*w^a (X-0.05), a recessive mutant known to be a specific modifyer of w^a, changing its phenotype towards wild type (GREEN, 1959 a).

2. suppressor of forked, *su-f* (X-64±), which is a specific enhancer of w^a, diluting its eye colour to almost white (GREEN, 1959 a).

3. zeste, *z* (X-1±), which has been found in males to specificly dilute the phenotype of w^a (whereas w^{ch}, w^e, w^h, and *sp-w* are not affected).

4. spotted-white, *sp-w* (X-1.5), the rightmost pseudoallele at the *white* locus, which in heterozygotes with *w*-mutants at the other subloci yields a complementary effect (LEWIS, 1956; GREEN, 1959 b).

The three first-mentioned genes were introduced in the chromosomes containing the reversions by means of crossing over. The gene combinations used for the analyses have been produced and tested in the following way:

A. *su-w^a*, *w^{a}R*

Heterozygous females of the constitution y^2 *su-w^a* w^a/w^aR were produced and the male offspring from these were scored for crossovers with phenotype y^2 in combination with the darker eyes characterizing the reversions. To test if crossing over had really occurred to the right of *su-w^a*, and not between this gene and y^2, the crossover males were individually crossed to w^a females, and the heterozygous daughters, presumably of the genotype y^2 *su-w^a* w^aR/w^a were backcrossed to their w^a brothers. The offspring was scored for y^2 w^a males, which would have been produced in about 1.5 % if *su-w^a* was not included in the genotype of the mothers. No such animals were found in any of the tests. Concerning the very light reversions R6 and R8 the method used might fail if the phenotype of *su-w^a* w^aR was not appreciably darker than that of *su-w^a* w^a itself. Indeed, this might have been the case for R8, which behaves very oddly in this combination. See page 623.

B. *w^{a}R*, *su-f*

From heterozygous females of the constitution w^a *su-f*/w^aR crossing over between the *apricot* locus and that of *su-f* would be expected in 50 %. Crossover daughters, from the cross to w^a *su-f* males, having the wanted constitution w^aR *su-f*/w^a *su-f*, were however phenotypically very similar to the non-crossover class w^aR+/w^a *su-f*. Individual daughters therefore were tested by outcrossing to w^a *su-f* males. The non-crossover females can after crossing over give rise to offspring with w^a phenotype, whereas the others only give two classes of offspring, the reversion type and the w^a *su-f* type, which is almost white in appearance. This method failed for R8, which gave no w^a offspring in the first cross, and thus

apparently no crossing over between w^a and *su-f*. The reason for this has not been further investigated.

C. *z*, w^aR

Heterozygous females of the constitution *sc z ec*/w^aR, were crossed to *sc z ec* males to produce the wanted crossover type. In the interval *sc—ec* three different types of crossing over can take place, giving rise to *sc* phenotypes, viz.: (1) $sc+w^aR+$; (2) *sc z* w^aR+; (3) *sc* $z++$. Type 2 is the wanted one. Among the male offspring type 3 can be discriminated as being real wild type in contrast to the others which contain the reversion and thus are lighter. In order to discriminate between type 1 and type 2, individual males with scute and reversion phenotype were crossed to homozygous *sc z ec* females. The daughters from type 2 then show the zeste phenotype, whereas those from type 1 are wild type.

When the right kind of chromosome had been obtained, the males containing it were crossed to *y f* attached-X females, to preserve it unchanged until the chemical analyses were undertaken. Newly hatched males were then collected. In the comparison involving *sp-w*, heterozygous females, $w^aR+/+sp\text{-}w$, were directly produced for the analysis.

All the animals used in the chemical analyses were bred simultaneously to avoid any seasonal differences in breeding conditions. They were cultured on standard cornmeal-molasses-agar-yeast medium at 25° C and were aged for 3—4 days before being used for the analysis. In some cases the flies were deep-frozen and stored in this condition until they were analyzed at a later occasion.

The quantitative determination of the eye pigments was made by the spectrophotometric method described earlier (RASMUSON *et al.*, 1960). 30 heads were extracted in 3 ml for each determination. The intensity was measured as the extinction (log I_o/I) in a Beckman DU spectrophotometer at 480 mμ for the red and 470 mμ for the brown pigments.

RESULTS

The results of the pigment determinations are shown in Table 1 and in Figs. 1—4. The original values are the amounts for reversions and w^a in wild type background. These values are partly identical with those given in the previous study but have in some cases been extended. Thus, for R1—R4, w^a and Canton-S male as well as female determinations are available, whereas R5—R8 are only analyzed as males. The

TABLE 1. *Estimates of red and brown eye pigments in different genotypes.*

The amounts correspond to a concentration of 10 heads per ml solvent, and are given as $100 \times \log I_o/I$. N=number of determinations, m=male, f=female estimate.

Red pigments

Mutant	Original value			In combination with							
				su-w^a		*su-f*		*z*		*sp-w*	
	N m	N f		N		N		N		N	
w^a	3		0.83±0.19	7	3.86±0.24	3	0.77±0.14	3	1.90±0.06	5	6.54±0.39
		5	3.52±0.15								
R1	5		25.48±0.65	10	17.18±0.60	5	9.98±0.27	5	7.92±0.24	5	36.62±0.70
		5	19.98±0.47								
R2	8		22.45±0.48	10	24.13±0,61	5	16.26±0.67	5	10.72±0.59	4	34.83±0.90
		5	21.74±0.73								
R3	6		5.72+0.33	14	12.80±0.33	5	1.36±0.18	5	2.04±0.16	5	14.83±0.40
		11	5.02+0.24								
R4	5		27.34±0.74	10	18.99±0.57	5	15.66±0.89	5	4.14±0.21	5	30.86±0.50
		5	19.18±0.67								
R5	10		15.74±0.46	10	32.62±0.66	5	11.20±0.47	5	8.70±0.24	5	36.08±0.31
R6	10		3.70±0.32	10	8.62±0.17	5	1.48±0.12	5	2.74±0.40	5	10.82±0.55
R7	9		20.79±0.45	5	28.36±1.35	5	11.00±0.22	5	7.42±0.39	5	35.20±0.68
R8	6		3.45±0.16	5	3.02±0.32	—	—	5	1.94±0.25	5	6.98±0.55
Canton-S	3		56.67±0.49								
		5	59.02±0.52								

Brown pigments

w^a	3		2.10±0.23	7	4.73±0.29	3	0.63±0.09	3	0.33±0.14	5	3.38±0.23
		5	1.82±0.07								
R1	5		10.14±0.48	5	9.26±0.35	5	5.50±0.19	5	6.22±0.20	5	9.04±0.40
		5	9.14±0.69								
R2	8		10.58±0.47	5	7.98±0.32	5	6.46±0.24	5	6.48±0.14	5	8.72±0.51
		5	12.34±0.82								
R3	3		6.94+0.41	9	7 92±0.33	5	1.68±0.23	5	2.82±0.14	5	5.36±0.12
		5	7.26+0.54								
R4	5		11.26±0.58	5	8.72±0.40	5	6.52±0.53	5	4.10±0.16	5	7.52±0.53
		5	11.46±0.45								
R5	5		8.22±0.31	10	9.41±0.26	5	5.02±0.18	5	5.72±0.23	5	7.96±0.40
R6	5		4.98±0.26	10	7.78±0.22	5	2.22±0.14	5	1.76±0.18	5	4.16±0.17
R7	6		9.03±0.55	5	8.92±0.31	5	6.68±0.34	5	5.72±0.23	5	8.54±0.17
R8	6		4.82±0.25	3	1.63±0.58	—	—	5	1.12±0.17	5	4.10±0.31
Canton-S	3		9.50±0.23								
		5	10.36±0.30								

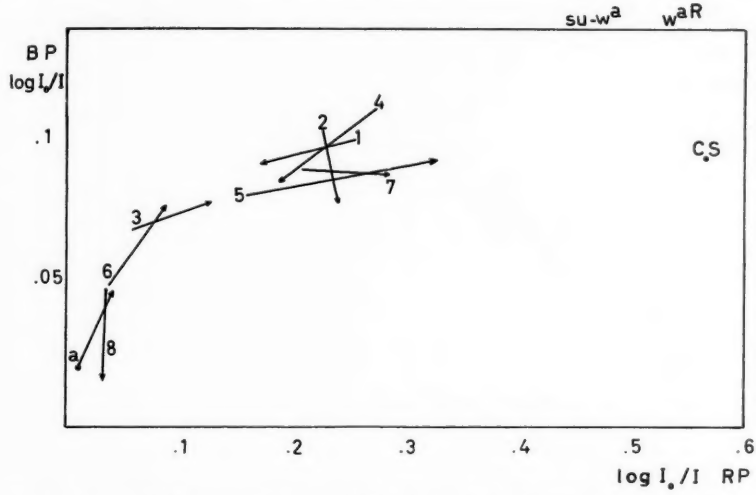

Fig. 1. Changes in the amounts of red (RP) and brown (BP) eye pigments caused by the interaction with *su-w*^{a}. The arrows go from the phenotypic values of the original genotypes to those of *su-w*^{a} *w*^{a}*R*, respectively *su-w*^{a} *w*^{a}. No. 1—8 stand for *w*^{a}*R* No. 1—8, a for *w*^{a} and CS for Canton-S. Estimates from males.

proper comparison is of course between animals of the same sex, and this has been done wherever possible. The only exceptions are the comparison with *sp-w* heterozygotes in R5—R8 where the original values are from males. It is not believed that this inappropriateness has in any way influenced the main results or the conclusions, since most of the sexual differences in eye pigments in the reversions are small. This is in agreement with the dose compensation known to prevail in the *apricot* locus (GREEN, 1959 a).

The interaction with *su-w*^{a} is shown in Fig. 1. This gene increases the amount of red pigment as well as that of brown pigment in the apricot phenotype and has a very erratic effect on the reversions. Some increase their intensity, others decrease, some are mainly changed in the red pigments, others in the brown ones. R6 and R8, which appeared almost identical in their original intensities are changed in quite opposite directions. There are however some doubts about the correctness of the R8 estimate. It appears from Fig. 1 and Table 1 that its intensity is less than that of the *su-w*^{a}, *w*^{a} type, but in the building up of the stocks the foundation animals were discriminated as being darker than this type (see page 620). When the records were put together this stock had been

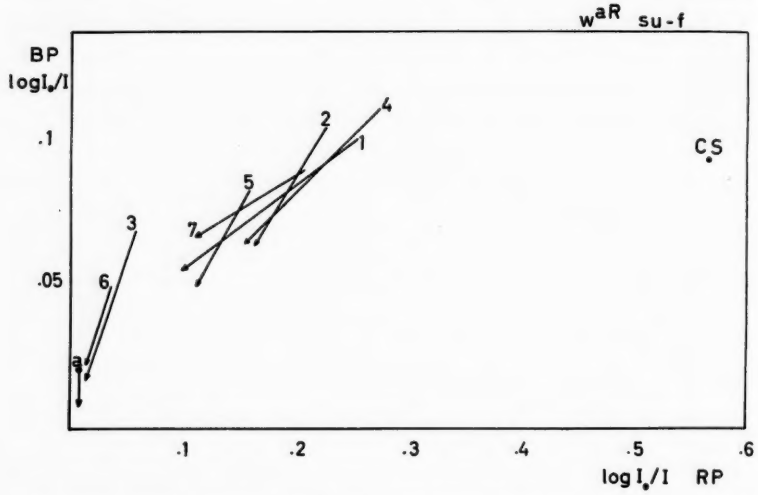

Fig. 2. Changes in the amounts of red (RP) and brown (BP) eye pigments caused by the interaction with *su-f*. The arrows go from the phenotypic values of the original genotypes to those of w^aR *su-f*, respectively w^a *su-f*. No. 1—7 stand for w^aR no. 1—7, a for w^a and CS for Canton-S. Estimates from males.

lost and the determinations could not be repeated. For all the other reversions the changes in eye pigmentation, caused by the introduction of the gene *su-*w^a, are significant, either in amount of brown, or in amount of red, or in both.

The introduction of the gene *su-f* changes the eye colour of w^a to almost white, and seems to have a similar diluting effect on all the reversions (Fig. 2). All changes in amount of red as well as of brown pigments are significant. R8 is not included in this experiment, since it was found to be impossible to introduce the *su-f* gene in the chromosome containing R8 (see page 620).

The reversions interact with *z* (Fig. 3) in a way which is very similar to that with *su-f*, but the diluting effect is generally stronger, and the decrease thus more marked. The darker reversions are drastically changed for both red and brown pigments, the less intense ones, R3, R6 and R8, mainly in their amount of brown pigments. Only w^a itself has experienced a small increase in the amount of red pigments. The difference between R6 and R8 in this combination is barely significant for the brown and not for the red pigments.

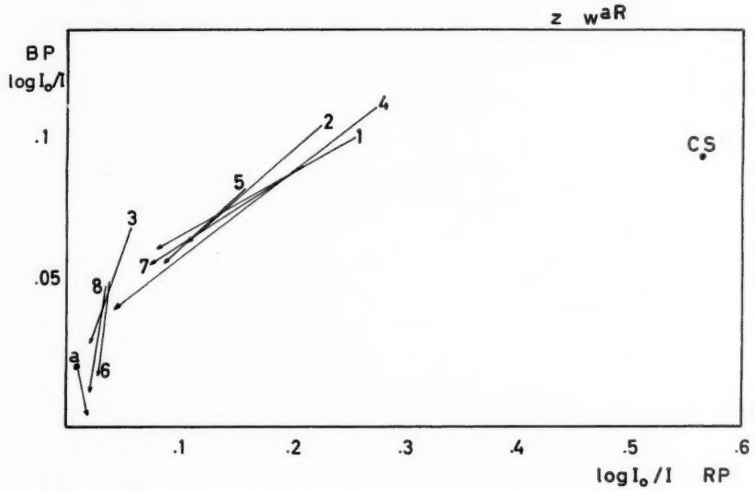

Fig. 3. Changes in the amounts of red (RP) and brown (BP) eye pigments caused by the interaction with *z*. The arrows go from the phenotypic values of the original genotypes to those of *z* w^aR, respectively *z* w^a. No. 1—8 stand for w^aR No. 1—8, a for w^a and CS for Canton-S. Estimates from males.

The complementary effect in heterozygotes with *sp-w* is shown in Fig. 4. It is peculiar in that w^a alone increases the amount of both red and brown pigments, whereas all the reversions agree in increasing the amount of red and decreasing the amount of brown pigments. The values for homozygous *sp-w* females, 1.87 ± 0.18 for red and 0.56 ± 0.08 for brown pigments (6 determinations), are also indicated in the figure. The complementary effect for *sp-w* and w^a is clearly demonstrated. The changes introduced in the reversions are significant in all cases for red and in R2, R3, R4 and R6 for the brown pigments. R6 and R8 differ in this combination significantly in the amount of red pigments.

DISCUSSION

The estimates of red and brown pigment intensities in the combinations of the reversions from w^a with the modifying genes *su-*w^a, *su-f* and *z* all differ from the amounts found in the original reversion genotypes in wild type background. Since one of the genes, *su-*w^a, is known to interact specificly with w^a and not with any other *w*-mutant, and since *z* as well as *su-f* enhance only the alleles of the *apricot* sublocus

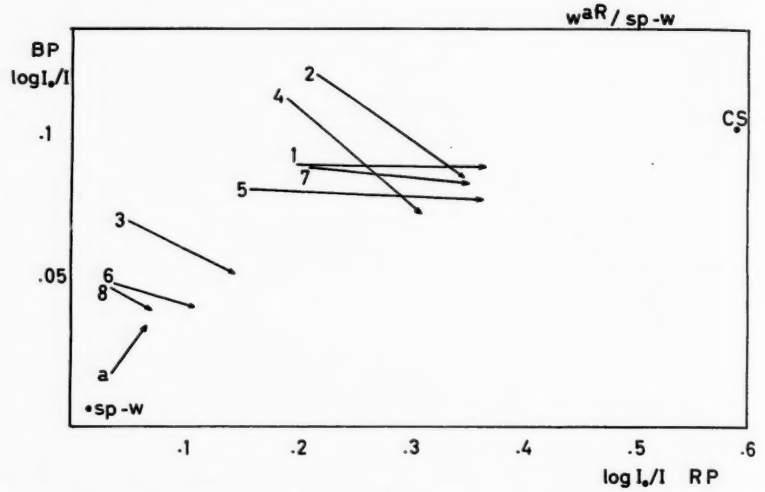

Fig. 4. Changes in the amounts of red (RP) and brown (BP) eye pigments caused by the complementary effect in heterozygotes with *sp-w*. The arrows go from the phenotypic values of the original genotypes to those of $w^aR/sp\text{-}w$ respectively $w^a/sp\text{-}w$. No. 1—8 stand for w^aR No. 1—8, a for w^a and CS for Canton-S. Estimates from females, except for the original genotypes w^aR No. 5—8.

(2′ according to GREEN, 1959 b), the all-prevading effect on the reversions emphasizes their close relationship with the foundation w^a mutant.

The study of the combinations with the interacting genes has discriminated further between the reversions. No identical results were obtained for different reversions, and thus no reversions can be supposed to be identical in function. The most similar among the reversions, R6 and R8, were found to interact very differently in combination with $su\text{-}w^a$. As has already been stated, the estimation for R8 in this combination is somewhat dubious, but also the combinations with *z* and *sp-w* reveal differences which infer that the reversions are not identical. The eight instances of backmutation from w^a thus are all different, if not in phenotypic appearance so in their interaction with other genes, and it seems to exist manifold possibilities for mutational changes of the w^a gene, which all result in a darker, more nearly wild type, phenotype.

Concerning the relationship between red and brown pigments in the different genotypes, the phenotypic expression is best characterized if

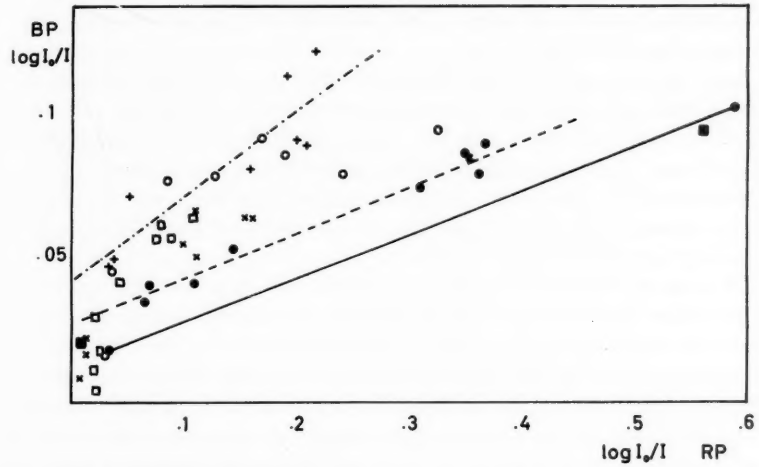

Fig. 5. Diagram showing the relationship between red (RP) and brown (BP) eye pigments in all the investigated genotypes. Linear regression lines for amount of BP on amount of RP are given for the original reversions (female estimates) and for the heterozygotes with *sp-w*. The linear relation between w^a and Canton-S is also shown (full line). + original reversions; ○ su-w^a w^aR; × w^aR su-f; □ z w^aR; ● w^aR/sp-w. Male (■) and female (●) estimates are also shown for w^a and Canton-S.

it is graphically represented as has been done in the figures. In Fig. 5 all the genotypes are put together in one diagram. As was shown in the previous study, the original reversions occupy a space which is not situated linearly between w^a and wild type, but deviates significantly in the direction of higher amounts of brown pigments.

Among the interacting genes, *su-*w^a has the most specific effect. There is no general shift of the phenotypes in a common direction, but merely an interchange of the reversions within their original space. Even disregarding the dubious effect on R8, it is obvious that the changes are unsystematic and unpredictable. It may be noted that *su-*w^a is able to discriminate also between w^a and w^{a2}, both mutants at the *apricot* sublocus and with similar phenotypes. The genotype, *su-*w^a, w^a gives a phenotype darker than w^a (Fig. 1), whereas *su-*w^a, w^{a2} is identic in appearance with w^{a2}. However, if a duplication of the nearby segment 3C1 (1′ according to GREEN) is introduced in the chromosome, also w^{a2} is suppressed by *su-*w^a (GREEN, personal communication). This may infer a structural difference between R1 and R4 on one hand, which are clearly enhanced by *su-*w^a, and R5, R6 and R7 on the other hand, which

are suppressed. This possibility will be further investigated by means of crossing over studies.

su-f and *z* interact with the reversions in a uniform and similar way. Both enhance the apricot phenotype and make the reversions lighter. As can be seen from Fig. 5 the interaction is not quite identical for the two genes, since the graphic interpretation shows their combinations with the reversions to occupy separate, although adjacent, spaces. It is clear that the modifying effect of *z* and *su-f* must be of a more general kind than that of *su-w*^{a}.

The same is true for the complementary effect in heterozygotes with *sp-w*. Here the effect, which is common to all the reversions, can be said to be normalizing, in that it changes the proportion between the amounts of red and brown pigments towards that found in wild type. However, there is still a shift towards an excess of brown pigments. The relationships are most clearly demonstrated by the regression lines drawn in Fig. 5. The equations for the regression of amount of brown pigments on amount of red is for the original reversions

$$Y=8.41+0.30\ (x—13.82)$$

and for the heterozygotes with *sp-w*:

$$Y=6.92+0.16\ (x—25.72).$$

The linear relationship between *w*^{a} and Canton-S, female values, is:

$$Y=6.09+0.15\ (x—31.54).$$

The total space occupied by the genotypes inferres a general relationship between amounts of red and brown pigments which is curvi-linear. The extended investigation of the gene functions in combinations of interacting genes thus has changed the view, which suggested itself after investigation of the original reversions only, viz. that the relationship was linear, but with a regression which differed from that indicated by the wild type and the foundation *apricot* mutant. A fundamental understanding of this relationship is not possible until the biochemical relations between the precursors of the two kinds of pigments are fully understood.

The mutational change from wild type to *w*^{a} involves a decrease in the production of brown as well as of red pigments, which are both very drastically reduced in the mutant. In the reversions from *w*^{a} the production of both kinds of pigments is repaired, and that of brown

pigments more completely so than that of red. The combinations between the reversions and the interacting genes results in modifications which are sometimes of the same kind as that found for w^a itself, sometimes of a different kind. Both *su-w*^{a} and *su-f* are phenotypically without discernible effect of their own, and the same is true for *z* in males. They may therefore be regarded as modifyers of the main gene, w^a or w^aR, as regards the eye pigmentation. It is possible to account for the interactions in a general way, if the modifying gene products are thought of as co-factors to the enzyme produced by the main gene. Providing these co-factors attach themselves at different positions of the enzyme molecule, their type of interaction can be due to the spatial specificity of this part of the molecule. *su-w*^{a}, in showing the greatest diversity in its interaction pattern, then should attach its co-factor in a position where the enzymes of the different reversions are most diverging, whereas *su-f* and *z* react with part of the molecules that are uniform in the reversions.

For the heterozygotes with *sp-w* this view cannot be applied. Here the complex problem of complementarity and non-complementarity of mutants within the same cistron is touched upon. The reversions represent a case of incomplete complementarity, and they behave uniformly in this respect.

SUMMARY

Estimates of the amounts of red and brown eye pigments are given for w^a and eight reverse mutations from w^a, R1 to R8, in wild type background and in combination with four genes known to interact with the phenotypic expression of w^a.

Three of the genes, *su-w*^{a}, *su-f*, and *z*, were introduced in the X-chromosome containing the reversion and the pigment estimates were performed on males. The fourth gene, *sp-w*, was studied in heterozygous females of the constitution *sp-w*/w^aR.

There was no identity among the reversions when all genotypic combinations were taken into consideration, and it can be concluded that they all represent unique mutational events.

The interaction between *su-w*^{a} and the reversions was irregular and the changes in phenotypic expression contradicting. It is known that the spatial arrangement of the *white* locus is of importance for the modifying effect of *su-w*^{a}, and it is suggested that the reversions may differ in this respect.

su-f and *z* both had a uniform and diluting effect on the reversions, but were not quite identical in function. The complementary effect in females heterozygous for *sp-w* was incomplete but brought about a normalization of the phenotype, viz. the relationship between amounts of red and brown pigments approached that of the wild type.

It is suggested that the irregularity or uniformity in effect of a modifying gene is due to the molecular structure of the enzyme produced by the main gene, to which the modifying gene supplies a specific cofactor.

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ON THE PRODUCTION OF THE TETRAPLOID HYBRID ASPEN BY COLCHICINE TREATMENT

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I. INTRODUCTION

THE term hybrid aspen is generally used to refer to the hybrid between the Eurasiatic *Populus tremula* and the American *P. tremuloides*. This cross was first performed in Sweden in 1939 (JOHNSSON, 1953). As the hybrid proved to be very fast-growing, other countries, including Finland, began to produce it. Although hybrid aspen is an interspecific hybrid, it seems nevertheless to produce fertile seed. But it is not to be expected that the superior gene combinations will be preserved in the offspring. Of course, it would be of great significance if this fast-growing hybrid could be made to reproduce sexually in such a way that the F_2 generation would be as fast-growing as the F_1 generation. Theoretically, at least, the amphidiploid hybrid aspen could be fertile, and since tetraploid plants are generally also larger and faster-growing than their diploid counterparts, it is very probable that economically, too, the amphidiploid hybrid aspen would prove to be advantageous. Moreover, this aspen will play an important part in the production of the triploid hybrid aspen, which seems to grow still better than the diploid hybrid aspen.

On the basis of these assumptions I produced the first tetraploid hybrid aspens at the Institute of Genetics of the University of Helsinki in 1959. I am indebted for advice and criticism to the Head of the Institute, Prof. ESKO SUOMALAINEN. The field work was performed in areas placed at my disposal by the Forest Officer, MAX HAGMAN, in the Forest tree breeding station at Ruotsinkylä, for which I here express my thanks. Financial aid for the study was given by Savo OY, by the courtesy of the executive director, Mr. LONDEN.

II. RESULTS

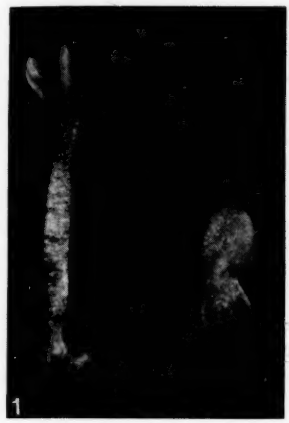
1. Colchicine treatment of the seeds

The seeds used had been shaken down in Ruotsinkylä on May 30, 1958, and originated from the cross female E 1095 Punkaharju, Finland (*Populus tremula*) × male U 2555 Ontario 43, 20 N (*Populus tremuloides*). The colchicine treatment was started in February, 1959. I chose lots of about 100 seeds, which were placed in Petri dishes on filter paper. The preswelling for an hour of the seeds was performed in such a way that the filter paper was moistened with an amount, say a, of distilled water. After an hour the same amount (a) of 0.2 % colchicine solution was put on the paper, the colchicine solution being thus diluted to 0.1 per cent. After the colchicine treatment, the seeds were washed in running water. The easiest way to perform this is to fold the paper into two and load the open edges with weights, thus preventing the seeds from being washed away by the running water. After 15 minutes' rinsing the filter paper was unfolded and the seeds spaced at suitable intervals with the aid of an artist's brush. The seeds were then allowed to germinate for 48 hours, the seedlings being then set in Jiffy pots and removed from the laboratory to a greenhouse.

When using the 0.1 per cent colchicine solution, I noted that the germination of the seeds was not impaired by the treatment, if this was preceded by swelling. On the other hand, only 7 per cent of the seeds germinated in a lot which was put into colchicine solution for 22 hours without preswelling. While the strength of the colchicine solution was kept constant, I experimented with various lengths of treatment from 2 minutes to 22 hours. The shortest treatment to produce the expected results was ten minutes. It seems, however, that an hour is the most suitable treatment time for the seeds of the hybrid aspen, provided the seeds are first swelled.

2. Comparison of the experimentally produced plants

The results of the colchicine treatment are already apparent 2 days after treatment, when the hybrid aspens have reached the seedling stage. The plants treated with colchicine differ markedly from the controls. A treatment of 10 minutes is sufficient for the production of these abnormal plants. The control plants are tall and slender, whereas those treated with colchicine grow low, thick and almost spherical (see Fig. 1). The cotyledons of the latter are of a darker green and clearly thicker than those of the control plants, the hypocotyl being very short, often



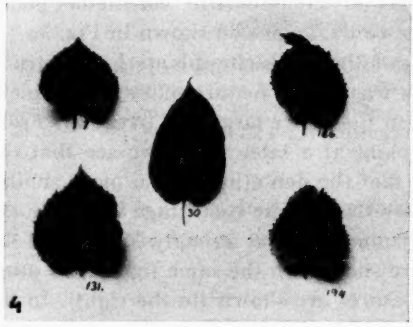


Fig. 1. Seedlings of the hybrid aspen, the one to the right treated with colchicine. — Fig. 2. A tetraploid plant produced by colchicine. Note the irregular shape of the first leaves. — Fig. 3. The diploid control plant no. 36. — Fig. 4. First leaves of the experimental plants. In the centre a diploid control (2n=38). No. 186, upper right, is a mosaic, epidermis containing 30, and the internal tissues, 51±5 chromosomes. The three other leaves are tetraploid.

only slightly longer than it is broad. The swollen hypocotyl generally distinguishes experimental seedlings after colchicine treatment of the seeds (WERNER, 1939). Similar phenomena were also observed by TANDON and BALI (1957) in connection with the colchicine treatment of *Linaria vulgaris*.

The mortality among these polyploid seedlings was very high during the first weeks, especially as they remain at the seedling stage from 2 to

4 weeks before the appearance of the true leaves. Most of the plants succumbed at this stage. This critical stage of the colchicine-treated plants also gave the control plants considerable advantage over the colchicine ones, and the value of this advantage was difficult to assess in later comparisons. A pause in growth was observed in other plants, too, after the treatment. For example, GOTTSCHALK (1959) reports that in polyploid seedlings of the tomato no development can be observed for 3—4 weeks after treatment. In the tomato, too, the interruption in growth is associated with a high mortality. GOTTSCHALK attributes the halt in growth to a toxic effect of colchicine. I am inclined to share this opinion since the growth of the hybrid aspens would be very good if the drawbacks of the toxic effect could be removed. A toxic effect is also indicated, in my opinion, by the fact that the first leaves of the colchicine plants are often quite asymmetrical; this asymmetry disappears later to such a degree that the last leaves in summer are often quite symmetrical. Fig. 2 shows an asymmetrical colchicine plant, which may be compared with the control plant 36 shown in Fig. 3.

In addition to the asymmetry of the colchicine plants I was struck by their peculiar dentation. This was also a feature of the above-mentioned plant 131. The teeth seen in Fig. 2 are large and irregular. From Fig. 4, which shows the same plant at a later stage, we see that the symmetry is almost perfect and that the dentation is also more similar to the normal, although still rather strong. The final stage is seen in the same figure in leaf 1, which is symmetrical but strongly toothed. In the following summer the leaves were similar. In the same figure two other colchicine plants with similar features are shown (to the right). In the centre of the figure a leaf of the control plant 30 is shown for comparison.

The peculiarity of these leaves suggested that the appearance of the plants would serve as a clue for identifying tetraploid individuals among the experimental plants. In 1959, therefore, I marked the plants which looked especially interesting. A later cytological examination of these plants confirmed the supposition that the appearance of the plants can be taken as a fairly reliable indication of polyploidy. Thus, the distinguishing signs are the asymmetry of the first leaves, which later disappears, a strong toothing of the tetraploid leaves, and a peculiar shape of the leaves, which are more rounded than in the control plants. The tetraploid leaves are also remarkably thick, feel smooth to the touch, and are dull and lighter in colour than in the controls. The veins are also very conspicuous. These features in the hybrid aspen indicate

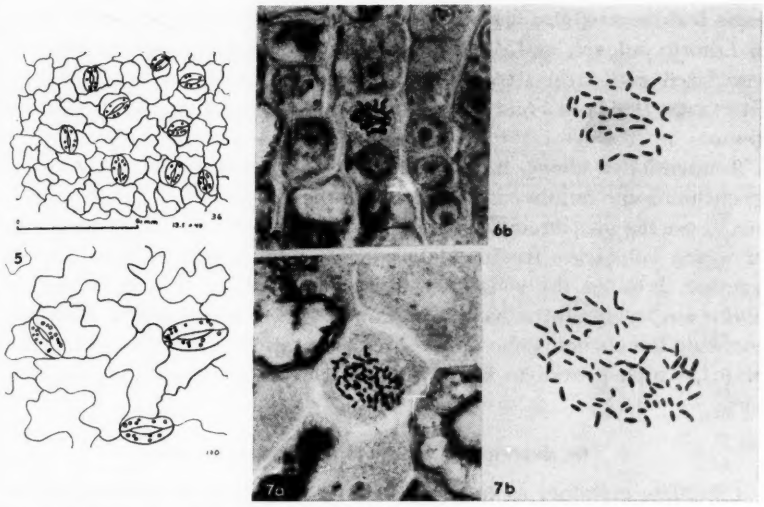


Fig. 5. Epidermal leaf cells at equal magnification, diploid above, tetraploid below. Fig. 6 a. Metaphase plate from a root tip of the diploid plant no. 30 (2n=38). Fig. 6 b. Drawing of the same plate. Fig. 7 a. Metaphase plate from a root tip of the tetraploid plant no. 170. Fig. 7 b. Drawing of the same plate.

that the plant is a polyploid. In the same way TIEMANN (1960) used the thickness of the leaves in searching for tetraploid individuals in his grass material.

3. Measurements of the stomata

An additional sign when polyploid individuals are sought among the hybrid aspens is the size of the stomata. The same phenomenon was observed with regard to grasses by TIEMANN (*l.c.*). One must, however, allow for the possibility that only the inner layers of the leaf are polyploid and that this phenomenon is consequently not seen in the stomata. In any case, the doubling of the chromosome number in the hybrid aspen also causes a clear increase in cell size, so that the tetraploidy, for instance, appears, already measured with the eye in the microscope, in the form of big cells. This is seen in Fig. 5, which shows epidermal cells from a leaf of the control plant no. 36 and of the tetraploid plant no. 170 on the same scale. From this figure it is also seen that there are smaller numbers of large stomata per unit area than small stomata. The

same feature was also observed by TANDON and BALI (1957 and 1959) in *Linaria vulgaris* and *Alyssum maritimum*, in which polyploidy was associated with large-sized stomata. Opposite results were reported by HERTZSCH (1951) in *Vicia villosa* and BALI and TANDON (1959) in *Iberis amara*.

As mentioned above, the increase in cell size caused by the increase in chromosome number is very remarkable. Therefore, it is advantageous to use the measurements of the stomata in order to find those plants in which colchicine treatment has led to an increase in chromosome number. I tested the reliability of this method by first marking the plants suspected on the basis of stomatal size to be polyploid, and then counting the chromosome number in preparations made from the same plants, which proved to have been correctly determined as polyploid.

4. The determination of chromosome number

Complete certainty as to the results of the colchicine treatment can only, of course, be attained by chromosome counts. I examined the chromosome number of the root tips and of the shoots. The Navashin-Karpeschenko fixation was used, after which the material was embedded in paraffin and sectioned at 10 μ. The sections were first stained in Feulgen and then in crystal violet.

With this method slides of the root cells of the hybrid aspen are obtained, from which the chromosome number can be accurately determined. This is the case at least so far as the diploid hybrid, with $2n=38$ chromosomes, is concerned (see Fig. 6). In tetraploid plants the possibility of erroneous counts is greater because of the larger number of chromosomes (76), the chromosomes of the hybrid aspen being small (Fig. 7). In the root metaphases examined, the colchicine treatment gave rise to tetraploids and also to aneuploids, which are grouped near either the diploid or the tetraploid number, as shown in Table 1. In all the root cells of the same plant, however, there was the same number of chromosomes, so that there were no chromosome mosaics in the roots. In 9 out of the 22 plants examined, the chromosome number of the root cells was tetraploid or nearly so.

The exact determination of the chromosome number in the shoots was far more difficult than in the roots, for the metaphase plates found in the former were not so clear as those found in the latter. An added difficulty was that in the same shoot both diploid and tetraploid cells might be present. In the leaves, for example, the mosaic structure was

TABLE 1. *Characteristics of 1+1-year-old plants.*

C=control plant, e=epidermal cells, s=parenchyma of the leaf.

Plant no.	Chromosome number		Changes in the morphology of the leaves	Large stomata	Height in cm	
	Root	Stem			1.10.59	1.10.60
1	39±1	76	x	x	41	74
5	39±1	38			16	13
48	70±2	38			38	102
62	36	38			51	115
66	37±1	38			32	42
68	76±2	38			40	122
70	78±2	38			50	111
83	76±1	38			31	56
90	82±2	38			32	90
98	—	38			21	23
102	—	38			24	38
109	76±2	38			29	97
110	—	38			41	50
121	—	38			42	77
130	38	38			51	122
131	37±2	76	x	x	44	91
145	—	e 76, s 38		x	5	5
148	—	38			31	40
156	—	38			7	12
157	42±2	38			33	40
166	37±1	38			6	8
170	76±1	76	x	x	3	42
183	38	38			48	134
185	73±2	e 76, s 38		x	27	41
186	—	e 30, s 51±5	x		37	94
194	74±2	72±4	x	x	38	74
201	42±2	38			51	144
216	38	38			25	46
262	38	38			58	124
264	38	38			54	118
270	—	38			22	28
C 9	38	38			37	50
C10	38	38			7	94
C19	38	38			24	23
C21	38	38			50	129
C24	38	38			24	124
C30	38	38			43	87
C33	38	38			39	90
C36	38	38			54	108
C37	38	38			20	25
C40	38	38			19	18

such that the epidermal cells of the upper and lower surfaces were tetraploid whereas the internal layers of the leaf were diploid. Converse cases were also found. For the present purpose, the most interesting plants are, of course, those with tetraploid shoots. As seen in Table 1, there were 4 of these among the 2-year-old plants.

Of all the plants treated with colchicine in 1959 there are only two hybrid aspens in which both the roots and the stems are tetraploid. In addition to these, the stems of two plants are tetraploid and the roots diploid. Both the above groups of plants are, of course, equally valuable, if one is merely trying to get a fertile tetraploid hybrid aspen. As Table 1 shows, there were a further 7 plants in which the root chromosome number was exactly or nearly tetraploid, but the stem was diploid or a mosaic of different chromosome numbers. These plants, too, will be used if a suitable method can be found for the vegetative propagation of the plants from the roots.

5. Growth of amphidiploids

One of the most interesting points to be studied is naturally the rate of growth of the amphidiploids produced. The saplings were planted out at Ruotsinkylä, where their overwintering succeeded well. Table 1 refers only to the plants surviving for 1+1 years. The measurements of these plants are presented for the autumns of 1959 and 1960. In comparing the amphidiploids with the controls, one must bear in mind that the latter had the advantage of earlier expansion of the first leaves. Hence it is probably more reliable to compare the amphidiploids with those colchicine-treated plants which remained diploid. Among these there were plants both taller and smaller than the amphidiploid ones. An accurate comparison is rendered all the more difficult as environmental factors exert a great influence on the growth of the plants. Thus, for instance, plant no. 170, which was amphidiploid throughout, grew only 3 cm in the summer of 1959, but began to grow very rapidly in the following summer until a plant disease destroyed its leaves at the height of the growing season. At this stage it was 42 cm tall. Nothing definite can be said as regards the growth rate of the amphidiploids at this stage, but it looks as if it will be at least satisfactory.

6. The plants produced in 1960

In the early months of 1960 I treated another lot of seeds of hybrid aspen with colchicine. This seed originated from the cross female *Popu-*

lus tremula E 1472, Tuusula, Lähdeniitty, Finland×male *Populus tremuloides* U 2566=T42, Canada. In the autumn of 1960 there were 115 plants of this lot surviving out of doors. The treatment of this material is still in progress, but measurements of the stomata indicate that the shoots of about 20 plants contain polyploid cells.

SUMMARY

(1) The seeds of hybrid aspen resulting from the cross female E 1095 Punkaharju, Finland (*Populus tremula*)×male U 2555, Ontario 43, 20 N (*P. tremuloides*) were treated with 0.1 per cent colchicine solution.

(2) Treatment for one hour can be considered effective if it is preceded by an hour's swelling.

(3) Even 10 minutes' treatment produces seedlings with short thick hypocotyls and thick cotyledons. The mortality among these polyploid seedlings was very high.

(4) Tetraploidy is evidenced by peculiarities in the morphology of the leaves.

(5) The increase in chromosome number causes a marked increase in the size of the guard cells of the stomata and in the size of the epidermal cells. There is a greater number of large stomata than of small ones per unit area.

(6) Both the appearance of the plants and the length of the stomata can be used as criteria in looking for amphidiploid individuals.

(7) The experiments produced both pure amphidiploids and mosaics in which the root was diploid and the stem tetraploid, or vice versa. The internal tissues of the stems showed mosaic configurations, but not the interiors of the roots. In addition, aneuploidy was found.

(8) The growth of the amphidiploid hybrid aspens produced in the experiments proved satisfactory. As, in addition, they are, theoretically at least, fertile, they may well find a practical application provided they grow well. Moreover, they will be of importance in the breeding of a triploid hybrid aspen.

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IMMERSION INTERFEROMETRY IN STUDIES OF THE FUNCTIONAL MORPHOLOGY AND CYTOGENETICS OF MOUSE ASCITES TUMORS

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I. INTRODUCTION

NEED for further means of determining similarities and differences between normal and malignant cells led to a consideration of the still relatively new, biological interference microscope. Concern here is with a quantitative morphologic approach to certain problems of functional morphology. With all the uncertainties of fixation, and of biochemical analyses of isolated cell components, the necessity for much increased utilization of living cells seems outstanding. Certain physical and quantitative information on the chromosomes could well give an insight into an interpretation of the behavior of normal and cancerous cells, in terms of chromosome constitution and structure.

By means of the interference microscope, invisible phase differences are changed into color or intensity differences, measurable with the same instrument. From these measurable values, the optical retardations, dry mass per unit area is calculated (BARER, 1956, 1959, for reviews). The accuracy is to a degree hitherto generally unknown (3×10^{-12} gm/cm^2 at 5460×10^{-8} cm green light). Such derivations are based upon the fact that mass per unit area is proportional to the difference between refractive index of the cell and water times the cell thickness; and knowledge that the refractive increment is fairly constant for all the different chemical substances of the cell.

From the work of BARER and his school, we know the probable limits of instrumental accuracy when the method is applied to several different biological systems. A great refinement in this field is represented

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by the special photoelectric scanning, recording, and integrating devices employed by CASPERSSON (CASPERSSON, CARLSON and SVENSSON, 1954). Still the meaningfulness of the limit of accuracy of the interferometric method in visualizing and measuring an experimentally useful variability in the cellular and chromosomal morphology is not so apparent. This introductory study on the Landschütz mouse ascites tumors is concerned with the application of this microscopic tool at the level of the cytological and cytogenetic problems currently being pursued.

II. MATERIALS, METHODOLOGY AND ITS INTERPRETATION

1. Materials and methods

Two lines of mouse ascites tumors routinely carried in the Cancer Chromosome Laboratory in Lund were employed in these studies. One was the hyperdiploid Landschütz tumor, originally obtained from Dr. GEORG KLEIN in Stockholm, and extensively analyzed by TJIO and LEVAN (1954). The second growth was an artificially-produced hypertetraploid strain of this tumor, obtained from Dr. RÉVÉSZ.

The Baker-Smith interference microscope was used with the Osram HBO200 mercury arc lamp and a Zeiss filter for isolating the 546 mμ wavelength green line, to obtain intensity patterns most sensitive for the human eye. Accessory equipment included the Baker fringe-field eyepiece, and the Baker half-shade eyepiece. For detailed descriptions of the construction and the manipulation of the interferometer, instrumental limitations and errors, theoretical considerations, and the empirical basis for determining dry mass from optical path difference, see three reviews on the subject (BARER, 1956; DAVIES, 1958; BARER, 1959), and a book by HALE (1958). For measuring the approximate refractive index of solutions used for mounting the cells, a sugar refractometer was used.

Only a few measurements were made, since the primary objective of this preliminary work was to determine first the extent of relative morphological variability in terms of dry mass from one point to another within the cell and among different cells; among the chromosomes of the same cell, and along the length of single chromosomes. Representation of variabilities was considered sufficient in most cases, and simply recorded photographically using monochromatic green light. Agepe orthochromatic film was used.

2. Interpretation and control of the interference-contrast image

The greatest limiting factor in the type of interference microscopy under consideration here is a geometrical one. Since light rays must pass through the portions of the cell underlying and overlying bodies within the cell, the absolute mass of an inclusion cannot be accurately determined. The optical path difference is the product of the refractive index and thickness of all material through a point in the cell. Unless the thickness of the object and the inclusion can both be determined, neither the mass nor the concentration of substance in the body can be calculated. However, the immersion method (matching the refractive index of the immersing medium and cytoplasm) can be used to distinguish between local variations in thickness, and variations in refractive index or concentration. Often considerable interest lies in the relative distribution of material, or differences displayed from one stage to another in the life cycle of the cell, which do not necessitate highly accurate, absolute determinations of the weights of inclusions.

The color or intensity with which the cytoplasm of the cell, or some part of the cell is observed in the interference microscope depends upon the optical retardation through that portion of the cell, relative to the immersing fluid in the reference area outside the object. The contrast with which the object is seen also depends upon the phase angle between the two interfering beams. This angle is changed by rotating a calibrated analyzer built into the microscope. Sensitivity will vary with the different types of contrast. Whereas in ordinary light microscopy, one observation under a single set of optical conditions should result in a good representation of all observable features, in interference microscopy it is not possible at one time to present all details observable by this method to best advantage. Some structure is best seen at one setting of the analyzer, hardly at all at others.

When the refractive index of the immersing fluid is equivalent to that of the cytoplasm, the cytoplasm is no longer visible against the background, except for its points of either higher or lower refractive index, which are still distinguishable. In the absence of the ground cytoplasm, study of this detail is facilitated. Moreover, when the refractive index of the medium is almost equal to that of the cell, the lens-like action of a spherical cell in phase or interference contrast is reduced or abolished, and internal contrast greatly improved. In the absence of a visible cytoplasm, nuclear detail is more easily observed. For a discussion of the principles underlying such phenomena, see the pioneer work of BARER and JOSEPH (1954, 1955 a, 1955 b and 1957).

For every degree of image intensity there are at least two possible values of optical retardation since, with retardations over 0.5 of a wavelength, intensity begins to fall again. So unless the maximum retardation does not exceed 0.5 wavelengths two parts of the cell having the same intensity need not necessarily have the same retardation.

III. INTACT, LIVING LANDSCHÜTZ MOUSE ASCITES TUMORS IN INTERFERENCE CONTRAST

1. Proper immersion of the living tumors for interferometry

The fact that the ascites tumors are free cells facilitates their use for interferometry. However, the density and geometry of these cells present problems of another kind, not encountered when using a less dense cell type, or a flat cell type.

Much of the methodology connected with the study of unfixed, living cells is concerned with the necessity of having an immersion medium for the microscope mounts which exerts the correct osmotic pressure, and yet has a refractive index suitable for a good differentiation of the cell, and for particularly that detail of the cell to be studied.

Several substances were experimented with as potential immersion media for the ascites tumors. To be useful as an immersion medium for interferometry, a substance must be capable of being varied in concentration to give a rather wide range of refractive index, and effect a minimum osmotic pressure, so that it can be made isotonic even at high concentrations. In the case of the thick ascites cell it is also necessary to have a medium in which the cell can be spread out to some degree, or in which it will flatten out a little without excessive squashing. The ascites cells were not washed free of the ascitic fluid, and a drop of the whole ascitic fluid with the cells was placed on the slide in the immersion medium.

(1) *Saline solutions:* Physiological saline has much too low a refractive index to serve generally as an immersion medium for the ascites cells.

(2) *Ficoll:* A synthetic, high molecular weight polysaccharide with a low osmotic pressure compared to sucrose (Pharmacia, Uppsala, Sweden). Ficoll can be dissolved in water to give a range of concentrations up to 50 per cent. The higher concentrations are viscous. An aqueous solution which reads 36 on the sugar refractometer exerts about the correct osmotic pressure. The ascites cells will not spread out sufficiently in those concentrations of Ficoll necessary for a suitably high

refractive index. The refractive index range of this substance is particularly wide though, and it can be useful in lower concentrations in combination with saline solutions for immersing tissue culture cells, or other cells types less dense or thinner than the ascites cells.

(3) *Dextran:* An anhydroglucose polymer with a low osmotic pressure. It is obtainable from Pharmacia, Uppsala, Sweden, in different molecular weight fractions. The highest molecular weight fraction was used here. Dextran is more viscous than Ficoll, and it can be dissolved in water to give a sugar refractometer reading only up to 10. The refractive index of this concentration is too low to be of much use for immersing the ascites tumors. For thinner, or less dense, or very flattened cells Dextran should prove useful.

(4) *Iodinated compounds:* Iodinated compounds of the type used clinically as radio-contrast injection medium are another category of potential immersing media. These compounds combine a high molecular weight with minimal toxicity of the iodine. One such substance is obtainable from Astra, Södertälje, Sweden, under the trade name "Umbradil", in vials in a concentration of 70 per cent. The 70 per cent solution of Umbradil strongly plasmolyzes ascites cells, and the solution must be diluted with distilled water to a concentration of about 10 per cent to give the proper osmotic pressure. Although of not much value for the ascites cells because of the inability to use higher concentrations without plasmolysis, Umbradil can be of use for mounting other cell types. It has the advantage of a low viscosity and freedom from contaminating particles, which can cause considerable nuisance in interference contrast.

(5) *Bovine blood albumin:* Fraction V bovine blood albumin from Armour proved very suitable for mounting the unfixed ascites cells. This is highly purified, and the least toxic substance found for immersing living cells, which at the same time has other properties essential for a mounting fluid for interferometric studies. This has been previously reported by BARER and JOSEPH (1955 a) for study of certain spermatocytes in phase and interference contrast. The albumin can be dissolved in water or in saline solution to give a concentration up to 50 per cent. Each 10 per cent concentration of protein has about the same osmotic effect on the mouse ascites tumors as about 0.07 per cent sodium chloride. For each 10 per cent of protein then, approximately 0.07 per cent less NaCl must be used in the saline solution in which the albumin is dissolved. A very wide refractive index range is obtainable by varying the relative concentrations of salt and albumin.

(6) *Gelatin:* Gelatin can similarly be dissolved in water or in some concentration of sodium chloride to give a mounting medium with a broad range of refractive index and the proper tonicity. Its usefulness for mounting ascites cells is about equivalent to that of the bovine blood albumin. It has the advantage of holding the cells in one spot on the slide, and the disadvantage of solidifying very easily.

For the general, overall interferometric pattern of the cell, an albumin solution which gives a reading of 30 per cent on the sugar refractometer dissolved in 0.7 per cent sodium chloride has a suitable, working refractive index for the visualization of internal cell detail. For study of the chromosomes, a slightly hypotonic solution is better, since it effects some spreading out of the chromosomes, and probably some disruption of the spindle. For this purpose, 30 per cent albumin dissolved in 0.65 per cent NaCl is suitable.

To prepare the microscope slide, first ring an area of an ordinary microscope slide and coverslip with vaseline. The albumin solution is put on the slide, and then the freshly-drawn ascitic fluid. The two are mixed with a fine glass needle, then the coverslip applied. All the excess fluid from under the coverslip is removed with a piece of filter paper. After a few minutes the cells are observed to be somewhat flattened out. The same amount of structure is observable in cells mounted in gelation solutions approximately equivalent in refractive index to the albumin solution.

Finding the most desirable concentrations for study of different cellular structure is complicated by the amount of spreading of the cytoplasm effected in the several solutions, and hence the amount of material lying above and below the inclusions which must be contended with. Further complicating factors are the general non-uniformity of the tumor population, and the differences in cytoplasmic concentration over the division cycle.

A concentration of albumin of 30 per cent seems high, but as much as one third of the fluid finally surrounding the mounted cells is comprised of the ascitic fluid, which has been reported to have a concentration of dry matter of only 3.9 per cent (KING, 1959). However the concentration of this fluid seems to vary considerably. If the concentration of the ascitic fluid is taken to be 4.0 per cent, the final concentration of material in the immersing fluid could be as low as about 13 per cent. There was some reversal of contrast among the cells studied here, and the optical path differences through parts of the cell were not always under 0.5 of a wavelength.

The tumors were not washed free of the ascitic fluid, because it was found that this had some slight adverse effect on the cells. For more strictly quantitative work, however, it would be necessary to know the exact concentration of the immersing fluid in which the cells are to be measured.

2. Interference-contrast images of the non-fixed, living tumors

In some non-dividing cells there is an accumulation of granular material lying in the cytoplasm to one side of the nucleus (Figs. 1, 2, 21, 23, 42), especially well demonstrated by Fig. 21. The few very large bodies must be fat globules (Figs. 2, 22). This region most likely represents the centrosome with its accumulation of surrounding material. Whether this organization of the cell is indicative of the stage of the division cycle, or the status of cell differentiation or degree of de-differentiation is not clear.

With the exception of a few other granules which lie at random, the cytoplasm of the metabolic cell, as observed in 30 per cent albumin solution, is free from granular material (Figs. 5, 22). The outermost portion of the cytoplasm has a quite different optical path from the innermost part (Figs. 4, 21, 23, 25, 42).

Details of the metabolic, non-dividing nuclei appear with unusual excellence of clarity relative to fixed and stained nuclei. In the "resting" cell, chromatin is mostly observed about the periphery of the nucleus (Figs. 1, 4, 6). Very fine threads are observed to run through the nucleus in some cases (Figs. 3, 4, 21). Possibly the largest percentage of the chromosomal material of these nuclei is not located about the nuclear membrane, as would first appear from such observations. Instead the enlarged bodies may represent only the chromocenters, or greatly enlarged heterochromatic segments of certain chromosomes.

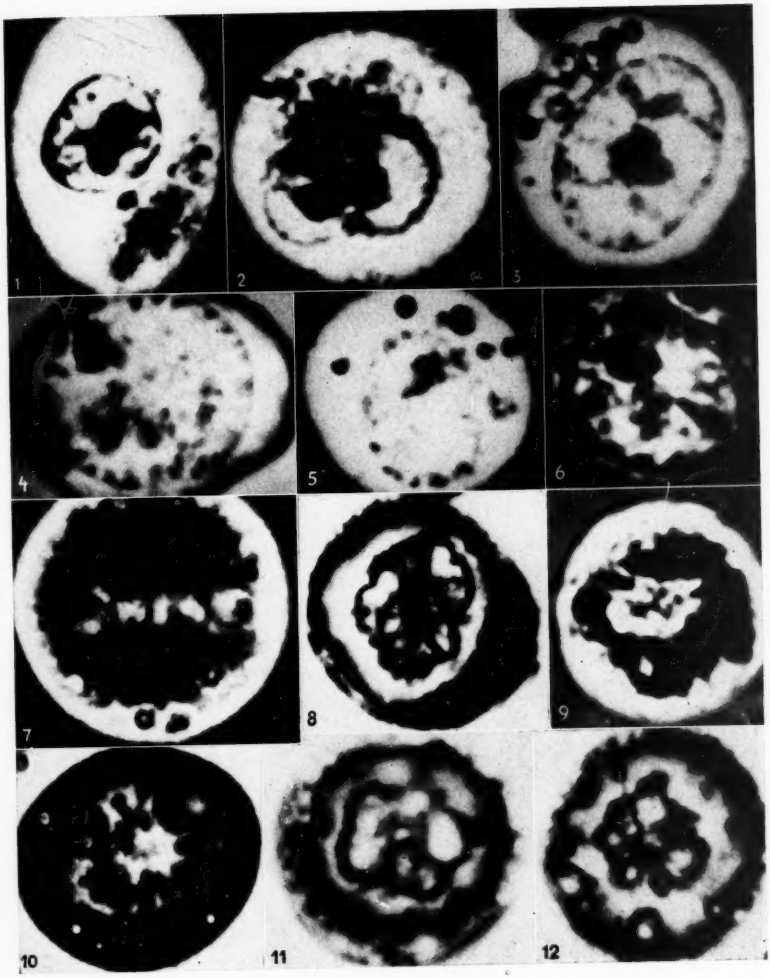
The nucleoli are very prominent, large, dense (Figs. 1—4, 21—24), and often contain one or several vacuoles (Fig. 3). There is always observed some nucleolus-associated chromatin (Figs. 3, 5, 23). In Fig. 23 the chromatin is the body of very minimal intensity lying across the nucleolus. The nucleolus in Fig. 23 is dark with its associated chromatin bright. In some cases a condensed, chromosome-like structure is also observed separate from the nucleolus. This body often appears markedly as if it were free in the nuclear sap (Fig. 23). Except for these bodies, the nucleoli, and the clumps of chromatin about the nuclear membrane, the nuclei in many cells appear empty.

By prophase, the heavy peripheral chromatin is no longer evident,

and throughout the nucleus the thickening strands of chromosomes with several points of greater density (Figs. 25, 27) are seen. The nucleus looks very much like that of a stained preparation. Fig. 26 shows a cell passing from the intermitotic stage into prophase. The mass of the whole area about the granular zone of the cytoplasm is differentiating from the surrounding cytoplasm. There is a gradient of optical path difference about this zone. By prometaphase, the spindle is rather well-formed, but still somewhat irregular in outline (Figs. 28—30). Fig. 28 shows a zone of small granules and larger fat droplets about the developing spindle.

The degree of density of the ascites cell, its spherical nature, the clumping and piling on top of one another of the chromosomes, make a very detailed analysis of the chromosomes in the living state impossible at either prometaphase or metaphase. Figs. 7 and 35 show side views of metaphase, and Figs. 8—12 and 31—33, polar views of the same stage.

Figs. 1—12. Landschütz mouse ascites tumors, hyperdiploid strain. Living cells mounted in 30 % albumin in 0.7 % saline. 100× shearing objective. — Fig. 1. Intermitotic cell. With the setting of the analyzer in which this cell was photographed an irregular nucleolar body shows in the center of the nucleus. Dense chromatin is present about the nuclear periphery. The cytoplasm appears homogeneous, except for an accumulation of granules in one area. Nucleus appears almost transparent except for the nucleolus and peripheral chromatin. Fig. 2. Intermitotic cell. In this cell the highly granular zone touches one side of the nucleus instead of lying some distance from it, as in Fig. 1. Fig. 3. Intermitotic cell. Large fat globules are seen at one side of the nucleus. Fine chromatin threads are barely observable as they run through the nucleus. A condensed, bipartite body, possibly a chromosome with small satellites, lies just above the nucleolus. At this setting of the analyzer, the nucleolus is easily distinguished from the associated chromatin which appears in darker contrast than the nucleolus. The nucleolus has small, less dense regions, most likely vacuoles. Fig. 4. Intermitotic cell. The cytoplasm has a different optical path about the edges of the cell. In this example, fine, fuzzy chromatin threads, distributed throughout the nucleus, and very dense peripheral chromatin, possibly chromocenters, are visible. Fig. 5. Intermitotic cell. Dark, irregular body in the nucleus is the condensed nucleolus-associated chromatin. Fig. 6. Intermitotic cell. Peripheral chromatin is highly retarding, and the nucleus large relative to the volume of cytoplasm. A little to the center of the nucleus is a dark-appearing nucleolus with a dense mass of chromatin lying across it. Fig. 7. Side view of a full metaphase plate. The spindle occupies most of the cell. Granules are collected about the periphery of the spindle. There is a rather sharp demarcation between the spindle and the rest of the cytoplasm. Fig. 8. Polar view of full metaphase. The spindle, as seen with reversed contrast from Fig. 7, is sharply defined against the cytoplasm. Fig. 9. Polar view of full metaphase with a spindle irregular in outline, though sharply defined. Fig. 10. Polar view of full metaphase with good perspective on the position of the chromosomes in the spindle of the unfixed cell. Fig. 11. Polar view of metaphase. The cytoplasm is differentiated into three areas. Fig. 12. Polar view of a metaphase having a very irregular spindle with large globules at its periphery.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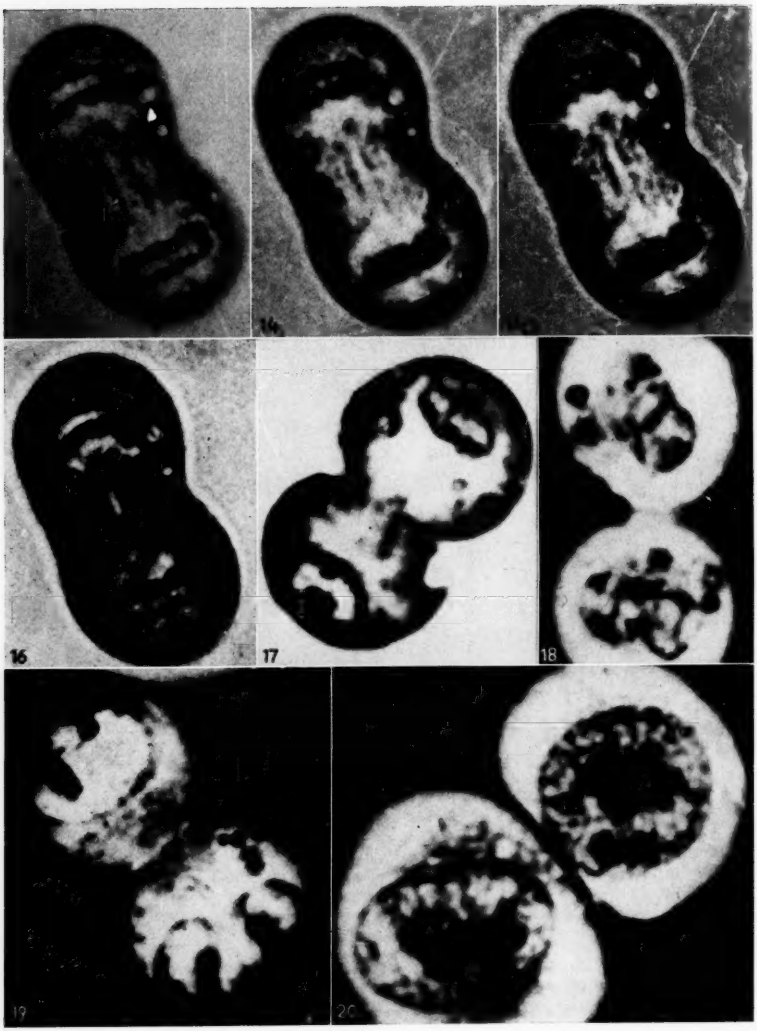
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The spindle is always optically clear and its periphery well-defined. At some settings of the analyzer, granular bodies can be seen at the periphery of the spindle (Fig. 12). At other settings, the cytoplasm at the metaphase is seen to be differentiated into three well-defined zones, the spindle proper, an intermediate zone, and the outer cytoplasm (Figs. 11, 33). The intermediate cytoplasmic zone is probably constituted of mitochondria, other small granules, and fat droplets. The anaphase and telophase spindle continue to be for the most part devoid of particles (Figs. 17, 36). Persistence during anaphase and part of telophase of the intermediate cytoplasmic zone described for the metaphase cell is demonstrated markedly in Fig. 36. Here the immersion method is used to distinguish between differences in the total optical path due to the geometry of the cell once it is partially flattened out on the slide, and real differences in the concentration of substances in the several zones of the spindle.

A kind of optical dissection of the spindle is seen in Figs. 13—16. A difference in density across this area is hinted at in Figs. 13 and 14 by an overlap of a greyish area onto the bright-appearing portion of the spindle. When the angle of the interfering beams is changed by rotating the microscope analyzer, most of the bright, central part of the spindle of Figs. 13 and 14 disappears, so demonstrating the inhomogeneity of the spindle mass (Fig. 15). The spindle immediately surrounding the telophase nuclei does not lose its contrast at this setting, as does this central part (Fig. 15). When the analyzer is further rotated, almost all of the spindle which appears bright in Fig. 14 disappears, and only the

Figs. 13—20. Landschütz mouse ascites tumors, hyperdiploid strain. Living cells mounted in 30 % albumin in 0.7 % saline. 100× shearing objective. — Figs. 13—16. The same telophase cell at different settings of the microscope analyzer. The borders between the spindle and outer cytoplasm are not so well defined as at metaphase. Note how the spindle extends behind the telophase nuclei. In Figs. 13 and 14 the spindle shows some variation in density, its most central portion having streaks of lesser intensity against the bright background. The rest of the spindle appears homogeneous. When the contrast is changed, a portion of the central part of the spindle disappears, while another part of it remains in view. Fig. 16 shows the last visible part of the spindle as the analyzer is further changed. Fig. 17. Late telophase cell. Remnants of the spindle persist. Fig. 18. Newly-cleaved cells. For observing nucleus only. Fig. 19. Newly-cleaved cells. Small granules are observable for some distance about the zone of cleavage. The cytoplasm is probably approaching its intermitotic differentiation of an inner and outer zone of different optical path. The two daughter nuclei have well-devoloped nucleoli. Nucleoli and associated chromatin are almost indistinguishable at this setting of the microscope analyzer. Fig. 20. Two daughter cells somewhat older than those of Fig. 19. Nucleolar configurations are quite dense. There is some accumulation of chromatin about the nuclear membrane by this stage. Threads of chromatin can be observed throughout the nuclei.

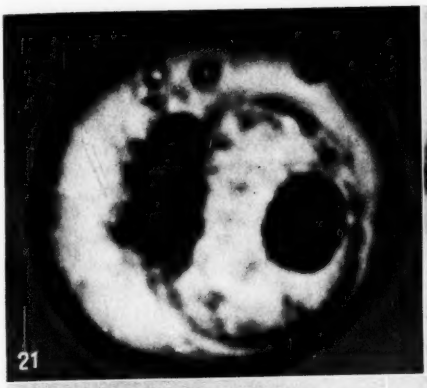
portion of intermediate intensity in the central region of the spindle of Fig. 15 remains in view (Fig. 16), seen now in reversed contrast, or bright instead of in intermediate intensity. If all of the mass of the spindle were optically homogenous, it would uniformly change contrast upon change of the phase, and it would disappear from view in entirety at the same setting of the analyzer.

Several stages of the cleavage process are seen in Figs. 17, 19, 37—39, 41). Remnants of the mitotic spindle are still seen at this stage. During cleavage there is a penetration of granules into the old region of the spindle (Figs. 19, 37—39). There is a special accumulation of small particles about the zone of cleavage (Fig. 19). At the telophase there must be a reorganization of the cytoplasmic material with its traces of the spindle, so that once again there is the division of the cytoplasm into a central and peripheral zone of different retardation. In the telophase nuclei, the nucleolar configurations can be observed developing as in the dark spots in the nuclei of Figs. 18 and 46. The heavy, peripheral chromatin is not yet observable. Fig. 19 shows two daughter cells with well-developed nucleoli. In Fig. 20 there are two daughter cells with fine chromatin networks. By this stage nucleolar configurations are well-formed. Blobs of chromatin begin to be seen at this time associated with the nuclear membrane, as in the lowermost cell of Fig. 20.

3. Discussion of the interference images of the living tumors

The separation of the cytoplasm of the mouse ascites tumor into two zones of different optical retardation has also been reported by KING (1959). Although the shape of the cell is a contributing factor, the difference is too well defined to be due to this alone.

Figs. 21—26. Landschütz mouse ascites tumors, hypertetraploid strain. Living cells mounted in 30 % albumin in 0.7 % saline. 100× double-focus objective. — Fig. 21. Intermitotic cell. The cytoplasm is divided into two zones, an outer one (black periphery of the cell), and an inner zone (bright center of the cell). The highly granular centrosome is in close contact with one side of the nucleus. Fig. 22. The centrosome and nucleus are seen in different contrast from that of Fig. 21, appearing here bright instead of dark. Fig. 23. Intermitotic cell. A chromosome-like body appears to be lying free in the nuclear sap. Two cytoplasmic zones are distinguishable. At the lowermost side of the nucleus there is a dark spot. This was observed to consist of small granules. Fig. 24. Intermitotic cell. A large, regular nucleolus, and not much chromatin visible. Fig. 25. Prophase. Contrast set for showing the condensing chromosomes throughout the nucleus. Fig. 26. Early prophase cell. The area occupied in the intermitotic cell by the centrosome is still differently retarding from the rest of the cytoplasm, with a gradient in optical path about this zone.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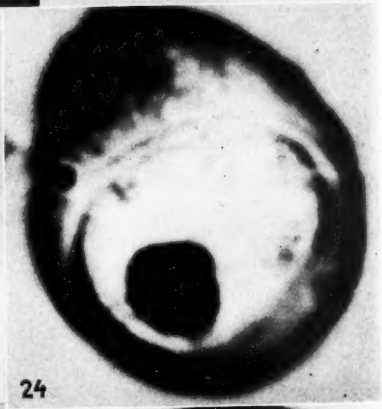
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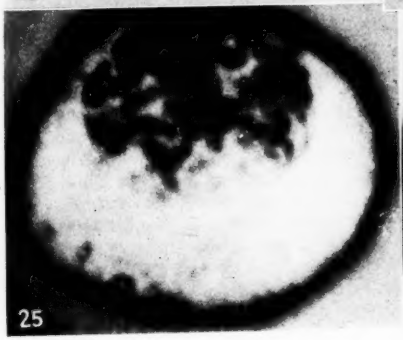
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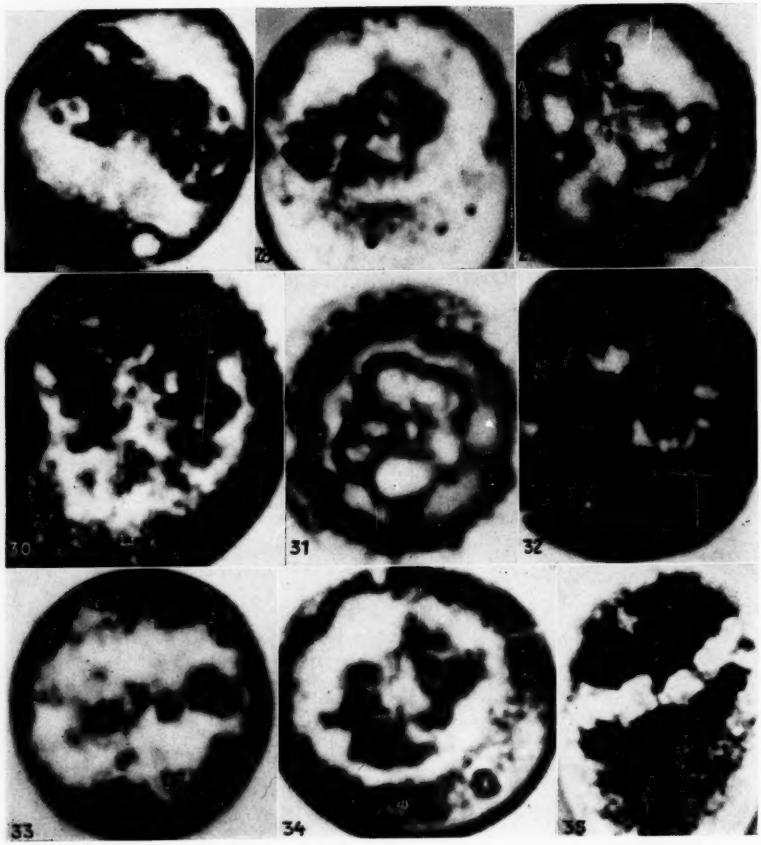
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When they are observable, the centrioles are certainly a sensitive indicator of the stage of the division cycle of the cells. Quite likely the mass of material which accumulates around the area of the centrioles, comprising with them the "cell center" or centrosome, is also a sensitive indicator of the time of interphase or post-telophase. The centrosome is also an expression of the differentiation process in certain cell types (see WILSON, 1928, for a discussion). The possibility that this organization of the cell might play a role in the de-differentiation process or in cancerogenesis has been mostly ignored in modern, experimental approaches, although COWDRY (1955) emphasized the importance of such studies. The collection of much of a certain portion of the granular component of the cytoplasm in one rather uniform mass enables a semiquantitative analysis to be made of this component in the single cell separately from the remaining cytoplasm.

Particularly because of the difficulties of fixation of the chromosomal material of interphase or non-dividing nuclei, detailed studies of such cells in the living state are highly desirable. Chromosomes in division stages must show only traces of any morphological or optical differences due to their functioning in the metabolic nucleus.

The chromosome-like structures which have the appearance of lying free in the nuclear sap of the interphase or metabolic nucleus may represent entire, condensed, allocyclic chromosomes. On the other hand, they may be particular chromosomal regions which undergo considerable enlargement during some phases of the life cycle of the cell. Their masses probably approximate that of a metaphase chromosome.

Whether or not these chromosome-like bodies, and the similarly-

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Figs. 27—35. Landschütz mouse ascites tumors. Living cells mounted in 30 % albumin in 0.7 % saline. Fig. 35 hyperdiploid strain, 100× shearing objective; Figs. 27—34 hypertetraploid strain, 100× double-focus objective. — Fig. 27. Early prophase cell. Granules and a large globule are lying at one edge of the central cytoplasmic zone, which appears mostly free of such particles. Fig. 28. Prometaphase. The developing spindle is seen as a still irregular zone of different optical path. Small granules and globules border the developing spindle, which appears mostly free of any such bodies. Fig. 29. Similar to Fig. 28. Fig. 30. A late prometaphase stage. Spindle is almost fully formed. Fig. 31. Same as Fig. 11. Fig. 32. Side view of metaphase. Contrast was set to show only the most central part of the spindle. Optical path through this portion of the spindle must be different from that through the rest of it. Fig. 33. Side view of metaphase with the three different cytoplasmic zones observable. Note how much of the cell the spindle occupies. Fig. 34. Polar view of metaphase with a more dispersed granular zone. Fig. 35. A metaphase spindle displaced to one end of the cell. The spindle appears quite optically homogenous, and the cytoplasm at one end of the cell very granular.

appearing chromatin associated with the nucleoli are the sex chromosomes or the so-called sex chromatin is open to question. Sex chromatin is known to persist in the condensed state in the intermitotic nuclei of a variety of mammalian tissues, and is absent in other tissues (BURLINGTON, 1958). Differences in behavior of the sex chromosomes or sex chromatin in the resting nuclei have been reported for the heterogametic and homogametic sex (REITALU, 1957 a; OHNO, KAPLAN and KINOSITA, 1958, 1959; OHNO and HAUSCHKA, 1960; YERGANIAN *et al.*, 1960). There has been a general failure to demonstrate the sex chromatin in old, well-established tissue cultures (ORSI and RITTER, 1958). The sex of the animal from which the tumor used here originated is not known, and the tumor demonstrates no sex specificity.

REITALU (1957 b) made a comparative study of the nucleoli and heterochromatin in the resting nuclei of mesothelial cells and the Landschütz hyperdiploid mouse ascites tumor. Taking into consideration the expected difference in appearance of fixed and living cells, results are in general agreement with the interference-contrast images. In normal, mesothelial cells, each nucleolus is in contact with one or more heterochromatic bodies and, in addition, there are several such bodies not associated with the nucleolus. In the cancer cells, the nucleolus is more irregular, and each nucleolus has several protruding regions from which attached chromatin tapers off into long, fine threads. In the malignant cell, there is a very striking increase in volume of the nucleolus, along with a greater exhibition of chromatin in the form of thin threads.

Little is known concerning the behavior of the mitochondria relative to the division cycle, although a great deal of information has been collected concerning their ultra-structure and biochemistry. BARER and JOSEPH (1957) made a study of mitochondrial behavior during meiosis in the spermatocytes of locusts, employing phase-contrast microscopy in conjunction with interferometry. A paper by ÖSTERGREN, MOLÈ-BAJER and BAJER (1960) mentions the movements of small granules in relation to a new theory on movements of chromosomes during division.

Figs. 36—41. Landschütz mouse ascites tumors, hypertetraploid strain. Living cells mounted in 30 % albumin in 0.7 % saline. 100× double-focus objective. — Fig. 36. Early telophase with the intermediate, granular zone between the spindle proper and the outer cytoplasm very sharply defined. Fig. 37. Late telophase with remnants of the spindle surrounding the telophase nuclei. There is some penetration of the granules into the region of the old spindle. Fig. 38. Similar cell to Fig. 37, but at a slightly earlier stage with more of the spindle still intact. Figs. 39—41. Cleaving cells with the cytoplasm once again being differentiated into two zones.

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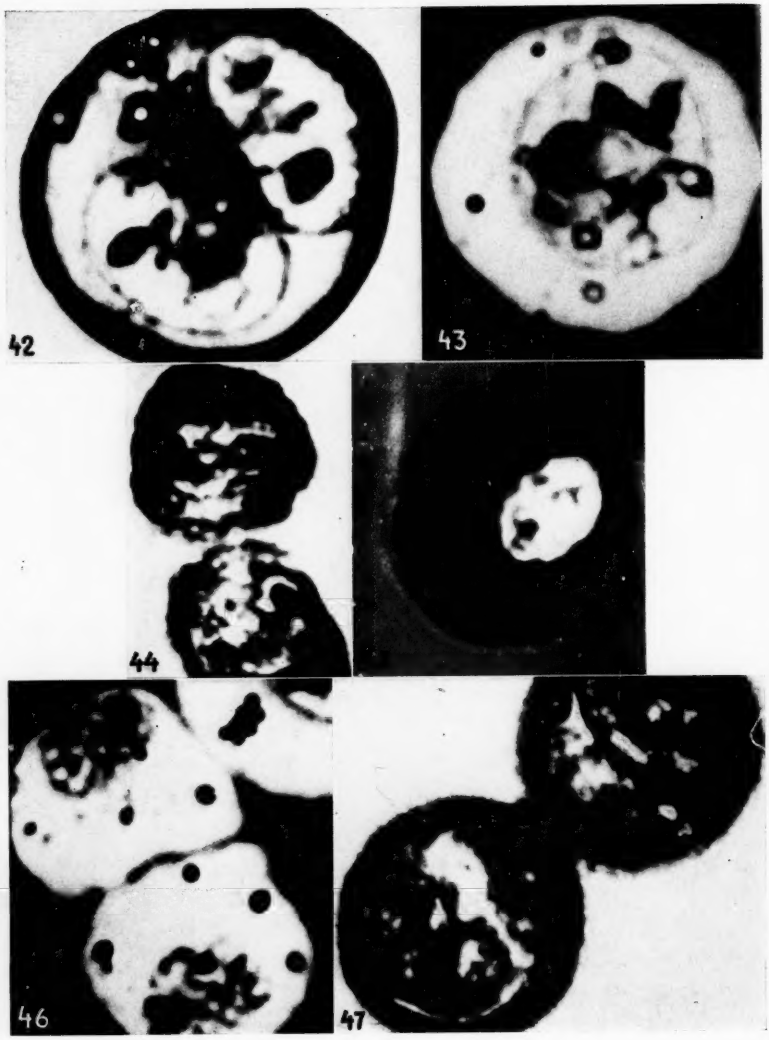
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In the case of certain anti-mitotic substances, BIESELE (1955) reported a possible interference with mitochondrial structure and function. Normally, mitochondria and other small granules are almost impossible to see, since they differ so little in refractive index from the surrounding cytoplasm. By combining techniques of refractometry and interferometry many of these particles can be differentiated.

In the unfixed ascites tumors, a differently retarding zone was not observed to circle the nucleus prior to the formation of the spindle, as reported by BAJER (1957) for living plant endosperm cells in phase contrast. In endosperm cells this differentiation appears about midprophase before the disappearance of the nuclear membrane. According to BAJER, this zone is mostly formed of cytoplasmic constituents, which mix with the nuclear sap to form the spindle apparatus upon the breakdown of the nuclear membrane.

On the basis of electron micrographs, SELBY (1956) reports that in the ascites tumors, the mitochondria migrate through breaks in the nuclear membrane, and become associated with the chromosomes at prometaphase. By metaphase they are removed from the center of the cell, and by prophase they are widely distributed throughout the cytoplasm.

IV. CHROMOSOMES OF THE LANDSCHÜTZ MOUSE ASCITES TUMORS IN INTERFERENCE CONTRAST

1. Preparation of chromosome squashes for interference contrast

For some purposes, the chromosomes can be examined at several stages of the mitotic cycle in the intact cell after fixation, or in the living cell immersed in a medium which will render the cytoplasm invisible. Generally, however, the detailed analysis of the chromosomes will have to be made from squashes of cells in metaphase which have been fixed,

Figs. 42—47. Landschütz mouse ascites tumor, hyperdiploid strain. Living cells mounted in 15 % gelatin in 0.7 % saline. 100× shearing objective. Fig. 42. Large, intermitotic cell with two nuclei, and the centrosome located between the two nuclei. Nucleoli and pieces of chromatin show with strong contrast. Fig. 43. Contrast set to show the nucleolar configurations. Fig. 44. Two young daughter cells with remnants of the mitotic spindle. Fig. 45. This could be a normal cell from the peritoneal fluid. Fig. 46. Daughter cells with developing nucleolar configurations. At this stage there is no heavy condensation of chromatin about the nuclear membrane. Fig. 47. Newly-cleaved daughter cell. There is some invasion of the granules into the central cytoplasmic zone.

as is done for light microscopic studies of stained chromosomes. The ease with which a chromosome preparation can be obtained will depend upon the density and size of the chromosomes, relative to the amount of cytoplasm. This will probably vary considerably from species to species, from tissue to tissue, and for different tumors and strains of tissue cultures.

For discussions concerning the refractive increment of fixed protein see BARER and JOSEPH (1955), and DAVIES (1958). Numerical values in the absolute sense perhaps have little meaning of immediate importance when obtained from material in which the total mass has been altered by the killing and fixing processes. Yet, even the altered weight of fixed structures can be meaningful when these are compared with one another over different stages of the life cycle of the cell; when the several chromosomes of the same cell are compared with one another; when different regions of the same chromosomes are considered; or the chromosomes from the cells of different tissues. For interferometric work the action of the fixative must be taken into careful consideration, and whether it results in an addition to the mass of the structure, or causes material to be extracted from it. BAKER (1958) presents a study of the action of fixatives. Chromosome fixation is considered here mainly in terms of the subsequent ease in making suitable chromosome squashes, a seeming necessity for the preliminary work.

Interference microscopic examinations were made of chromosome squashes of the Landschütz hypertetraploid mouse ascites tumor following treatment with several fixatives. The preservation of cytoplasmic structure and hardening of the cell with some fixatives render a good chromosome squash impossible, and any detailed chromosome-to-chromosome analysis is then not feasible. Fixation in Carnoy's fluid permits reasonably good squashing of the cell.

Fixation in 60 per cent acetic acid, as used by many authors, *e.g.* LEVAN and HAUSCHKA (1952), proved best for detailed chromosomal studies. The orcein stain is, of course, omitted from the fixative. The acetic acid serves well as a fixative here, because it clears away certain cytoplasmic structures, which would otherwise obscure the optical path difference of the chromosomes. Likewise, some of the cytoplasmic mass is dissolved in the subsequent handling of the slides, making for a still clearer chromosomal pattern. The acetic acid also swells the cytoplasm, leaving it soft and easily spread about the chromosomes.

From 15 to 18 hours prior to the sampling of the ascitic fluid, the mice were given 0.2—0.4 c.c. of 0.003 per cent colchicine in aqueous

solution intraperitoneally in order to produce colchicine-mitosis with its accumulation of metaphase figures. After sampling the fluid, the cells were pretreated with 1.12 per cent sodium citrate for 5—8 miuntes at room temperature while still in the ascitic fluid, to soften the cells and facilitate easy, uniform spreading of the cytoplasm. The mixture was centrifuged, the fluid poured off, and 60 per cent acetic acid added to the tubes containing the cells.

A drop of the acetic acid with the cells suspended is placed on the microscope slide, a plastic coverslip put on, and the cells squashed. The chromosomes can be examined with the 60 per cent acetic acid serving as the immersing medium, but they do not show with much contrast against the background, since the optical retardation through the chromosome is not much greater than through the surrounding acetic acid solution. The coverslip is sealed with rubber cement, then dissolved in acetone and acetone-xylene, 1:1 (ÖSTERGREN's modification of MELANDER's method with soluble cover slips; MELANDER, 1948; ÖSTERGREN and HENEEN, 1962).[1] The cells were mounted in 45 per cent acetic acid, 25, 15 and 5 per cent, and in water. The chromosomes were examined in all of these mounts.

The refractive index of 45 per cent acetic acid is still too high to give a good contrast for the chromosomes. For the chromosomes of this tumor, 5 per cent acetic acid and distilled water have the most suitable refractive indices (refractive index of water, 20° C, 1.332). This will vary depending upon the size and density of the chromosomes. Some swelling of the chromosomes was detected upon mounting in water and in the lower concentrations of acetic acid. For this reason, the chromosomes were further hardened for one-half to two hours in a 10 per cent solution of formalin before mounting in either the 5 per cent acetic acid or in water. Subsequent work has shown that methanol (refractive index 1.331, 20° C) can be used here in the place of water as the mounting fluid. In some cases dry mounts of the chromosomes seem more desirable.

Only those cells in which the chromosomes lie in a uniformly well-flattened cytoplasm can be used for examination, and preferably only those in which the cytoplasm is extremely well flattened out. If the chromosomes do not all have equivalent amounts of surrounding cyto-

[1] The plastic sheeting, Astralon, from which the coverslips were cut out is produced by Dynamit Nobel A.G., Abteilung Kunststoff-Verkauf, 22 c Troisdorf, Köln, Western Germany.

plasm, their optical paths will differ due to this, and real variabilities in the optical paths of the chromosomes will be more difficult to detect.

Care must be taken that chromosomes from two different metaphase groups are not mixed in the squashing process, and subsequently analyzed as belonging to the same cell. Due to the contraction process, and most likely also to chromosomal physiology during the division, chromosomes at slightly different stages may present wide differences in their optical paths. No more pressure should be put on the cells during the squashing process than is really essential, and only a small number of cells can be mounted on the same slide. It is not likely that the chromosomes of the same cell will receive different degrees of pressure. Moreover, the chromosomes appear to have an elastic-like quality.

In interpreting the interference-contrast images, and in observing the photomicrographs of the chromosomes, it is necessary to consider several points peculiar to the interference images. Only those chromosomes which are lying free should be considered, as one chromosome lying across another will affect the optical retardation through the region of the overlap. If the metaphase chromosome is lying on its side, the optical path will be through two chromatids instead of one, and consequently greater. Chromosomes or portions of chromosomes which are not lying flat must also be discounted, since light passing through them has a greater optical path due to the angle through which it had to pass. In the ideal chromosome plate, most of the chromosomes are lying free of one another and flat.

Since the chromosomes of the mouse ascites tumors retard the light under 0.5 of a wavelength, any point on the chromosome appearing brighter than others when the background is black, and any point darker than other chromosomal segments when the background is made bright, will be more highly light-retarding.

When the chromosomes differ much in their optical retardations from one to the other, the setting of the microscope analyzer must be different for showing, with maximum contrast, the various chromosomes of different phase retardations. Often, when the contrast is set for showing some one chromosomal variation, other variations, even in the same chromosome, will show with less clarity or hardly at all, depending upon the relative retardations. This sometimes gives the impression that some chromosomes are out of focus when they actually are in focus.

In much spread-out chromosome plates, there can be some overlap of the ghost image onto the real image, even with the 40× Baker shearing

objective. Care must be taken not to measure a chromosome which has the ghost image of another chromosome impinging upon it. (With the less easily manipulated Cooke-Dyson interferometer, objects with much greater diameter can be measured, so there would be no such difficulty with the ascites cells, and their chromosome plates.) This is more of a problem with the 100× Baker shearing objective than with the 40× shearing objective. Often only about half of the image is of use for accurate measuring with the 100× shearing objective.

2. Interference-contrast images of the colchicine-metaphase chromosomes of the tumors

On the average, a colchicine-metaphase chromosome of the Landschütz tumor retards the 546 green line about 0.1 wavelengths.

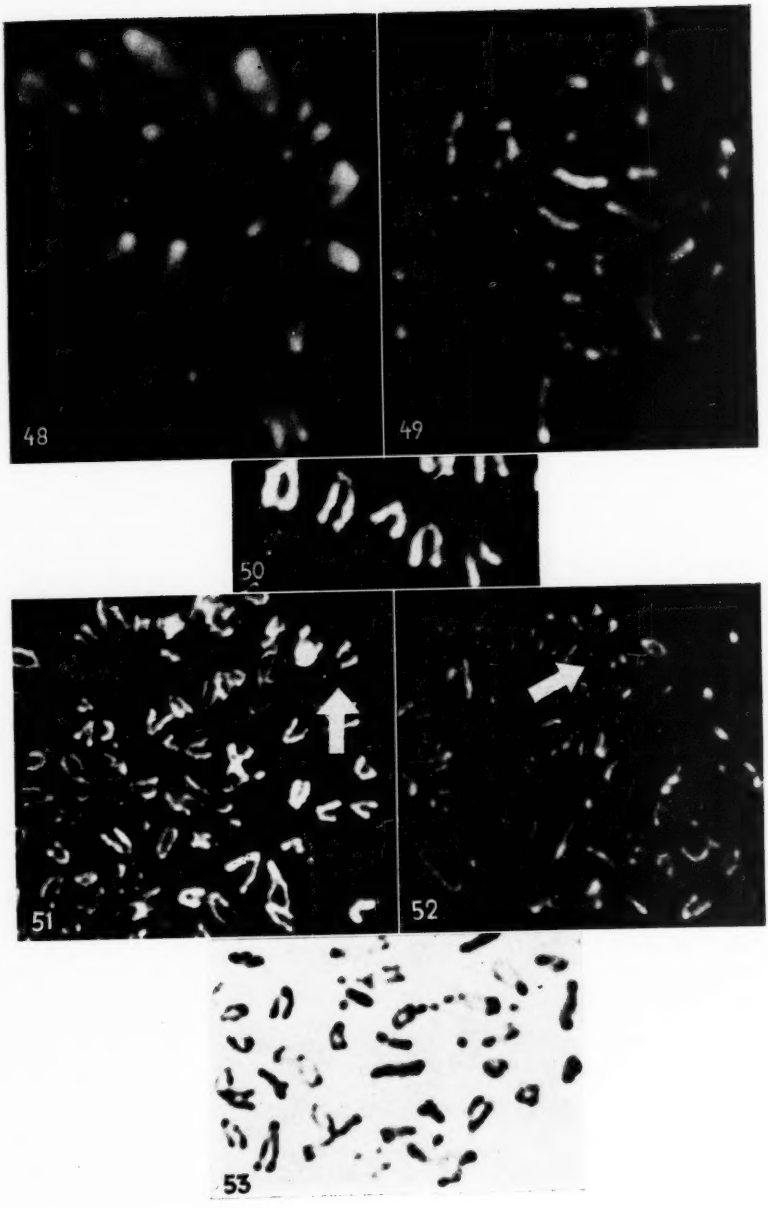
In some of the early metaphases of colchicine-mitosis, almost all the chromosomes show a greater retardation at either or both their proximal and distal ends, as in Fig. 48, where the terminal regions show with brighter contrast against the background than do the rest of the chromosome lengths, and with darker contrast against a bright background, as for some chromosomes in Fig. 53. The proximally differentiated regions must correspond either to the centromere proper, or to segments of heterochromatin known to be associated with the centromere in many species. The distal differentiation probably corresponds to the terminal, heterochromatic regions called telomeres in classical cytology (see LIMA-DE-FARIA and SARVELLA, 1958). In some chromosomes, this distal differentiation is of no greater size than the centromere itself, as is the case for a few chromosomes in Fig. 53, and the chromosome at the lower right-hand side of Fig. 49. Also, see the chromosome at the arrow in Fig. 52. In other cases, this terminal, dense zone extends for some distance along the length of the chromosome (Fig. 48). Fig. 50 shows a group of five chromosomes, three of which appear quite uniform in optical retarding properties all along their lengths, and two of which show variability at the centromeric portion (second and fifth chromosomes from the left). The centromeric end of two of the chromosomes of Fig. 50 is less light-retarding here than the remainder of the chromosome length. This is also the case for the chromosomes at the arrow in Fig. 52. The distal regions here show no difference in phase retardation from the intermediate lengths. The chromosome at the arrow in Fig. 52 has a greater retardation at the distal end of both chromatids than along the remainder of its length. The chromosomes

in Fig. 48 are all lying approximately flat. Not all of them show with the same contrast. In Fig. 49 some of the chromosomes are lying at angles, and in others the optical path is through both chromatids, as for example the chromosome in the lowermost portion of this plate.

In the same early metaphase group then, there are chromosomes which are entirely uniform in their optical path, alongside chromosomes with either proximal or distal differences, or both (also see Fig. 60). In some cases, the proximal region has a greater retardation than the non-terminal portion of the chromosome, and in other cases, less. The distal end of the chromosome was never observed to be less retarding than its intermediate length. The terminal differences in optical path extend to variable distances along the chromosome arm, not all the chromosomes of a cell displaying equivalent lengths so differentiated.

In a few metaphase groups, there is considerable lack of uniformity in the optical path differences through the several chromosomes (Fig. 59). Some of the chromosomes in Fig. 59 appear quite dark at this setting of the analyzer, while others show very faintly (due to their very different optical retardations). More often, the differences are much less exaggerated, and only a few chromosomes show in brighter or darker contrast than others of the same group, as in Figs. 54, 55, 58,

Figs. 48—53. Chromosomes of the Landschütz mouse ascites tumors, hypertetraploid strain, at colchicine metaphase. Sodium citrate pre-treatment, fixation and squashing in 60 % acetic acid, and hardening in formalin. Mounted in distilled water. All photographed with 100× shearing objective except Figs. 49, 51, and 52, which were photographed with 100× double-focus objective. In some cases the ghost image was cut off and is not shown here. — Fig. 48. Early metaphase or prometaphase chromosomes. Observe the chromosomes along the lower half of the plate. The proximal and distal ends of some of the chromosomes are more highly retarding than the remainder of the chromosome length. These regions extend for variable distances in along the chromosome. In the same group there are three chromosomes of completely uniform retardation. Fig. 49. Consider only the few chromosomes lying flat. Relative difference in optical path through the central and terminal regions of the chromosomes is greater here than in Fig. 48. Fig. 50. The first, third, and fourth chromosomes from the left appear quite uniform in optical path along their entire length. The second and fifth chromosomes show a lesser optical path difference in the centromeric region. Fig. 51. Disregard most of the chromosomes, as they are not lying flat. Even so there are differences in optical path from chromosome to chromosome, and along the length of a few chromosomes. Chromosome at the arrow has a greater optical path for some distance along its distal end, while the centromeric end shows relatively little retardation. Fig. 52. Much the same as Fig. 51. The chromosome at the arrow shows more retarding, knob-like distal ends, with a centromere undifferentiated from the rest of the chromatin. Fig. 53. Proximal or distal chromosome ends, or both, are strongly differentiated on the basis of optical path. Almost all of the chromosomes in the group suitably oriented for study show these differences.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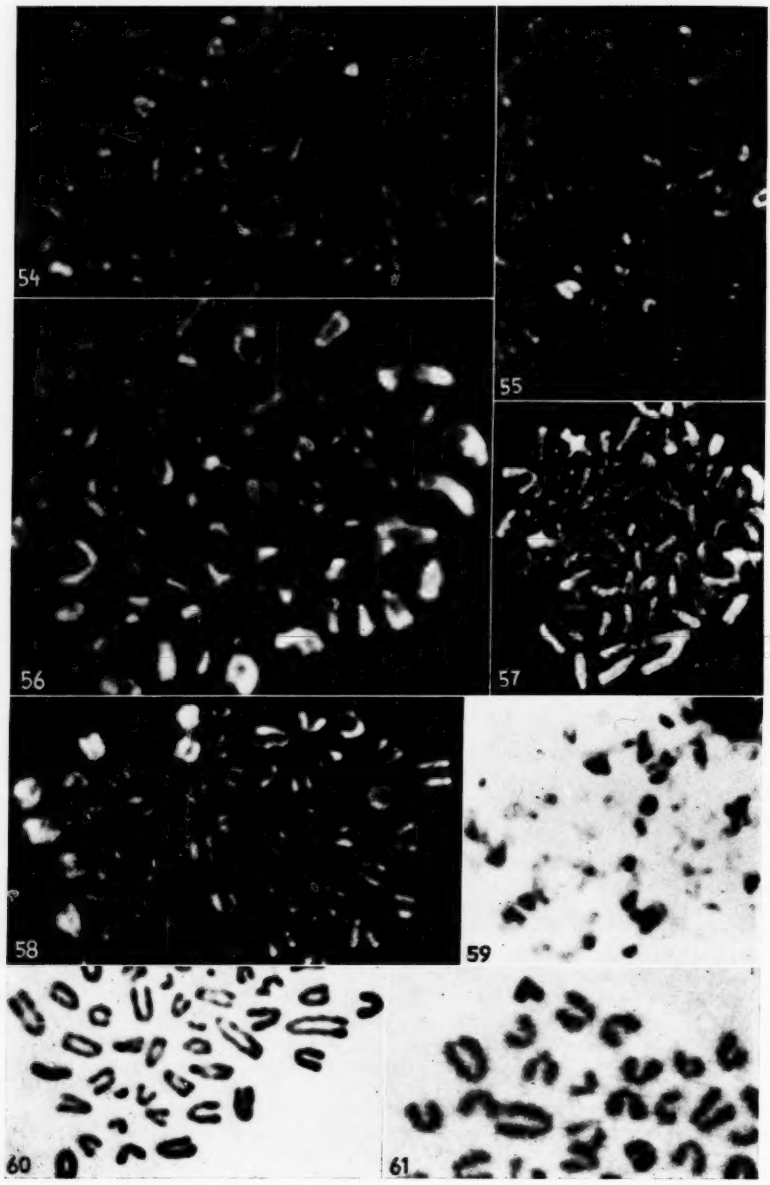
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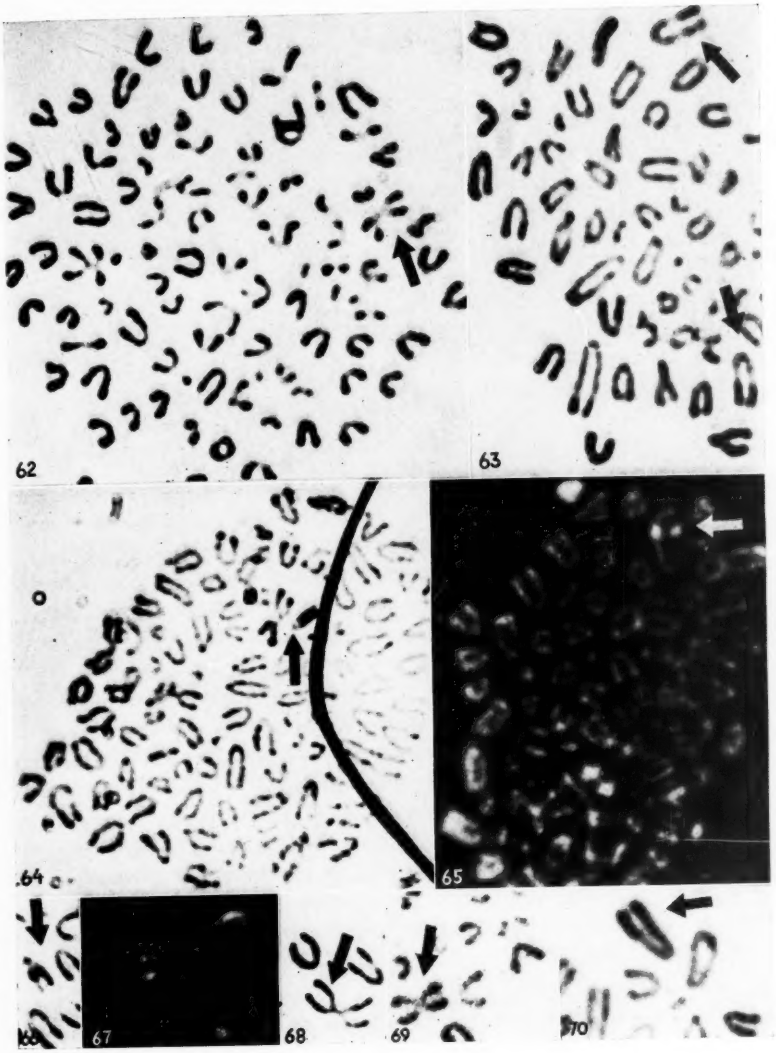
61, 65 and 72. In Fig. 54, retardation through the two chromosomes of maximum intensity lying across the top of the plate is obviously different from the retardation of the rest of the chromosomes of the group. There are two other chromosomes which appear bright because the light is passing through two chromatid thicknesses (upper and lower left side of the plate). In Fig. 55, there are two intermediate-sized chromosomes and two very small chromosomes at maximum intensity, which could be two different homologous pairs. In Fig. 61, at least two chromosomes appear with less intensity than the rest of the group (change of the contrast here, so that the background is bright instead of dark). In Fig. 65, the uppermost chromosome in the group has a greater intensity at this setting of the phase than most of the remainder of the chromosome group. Especially interesting in Fig. 64 is the chromosome at the right of the arrow, which is at minimal intensity all along its length with the exception of its centromeric region, which shows with a near maximum intensity. This chromosome is quite differently retarding from most of the remainder of the group, with the exception of the chromosome at the left edge of the plate just below the metacentric chromosome, and the uppermost chromosome, just near the line dividing the ghost image from the real image (see further, below). In Fig. 72 (a plate treated with lanthanum chloride, an electron stain) one chromosome shows in a brighter contrast than the other chromosomes.

Figs. 54—61. Chromosomes of the Landschütz mouse ascites tumors, hypertetraploid strain, at colchicine metaphase. Sodium citrate pre-treatment, fixation and squashing in 60 % acetic acid, and hardening in formalin. Mounted in distilled water. All photographed with 100× shearing objective except Figs. 56—59, which were photographed with 100× double-focus objective. In some cases the ghost image was cut off and is not shown here. — Fig. 54. Two chromosomes in the upper, left-hand side of this plate show with greater contrast against the background than do the rest of the chromosomes. Do not consider the chromosomes lying on their sides. There are smaller, less obvious differences in optical path among the other chromosomes. At this analyzer setting there is a slight indication of the differentiations at the terminal ends of the chromosomes. The ghost image covers part of the right half of the photomicrograph. Fig. 55. Three, and possibly four, of the chromosomes have a different optical path from others in this group. Figs. 56—57. Most of the chromosomes about the periphery of these plates have a different retardation from the rest of the chromosome group. This is particularly evident at one side of both of these plates. Fig. 58. Six of the chromosomes at one edge of this plate differ in optical path from the other chromosomes, which are not seen in good contrast at this setting of the analyzer. Fig. 59. A few of the chromosomes differ greatly in optical path from the others, which are hardly observable at this setting of the interfering beams. Fig. 60. Again the chromosomes at the periphery of the plate retard the light to a different degree than the others. Fig. 61. At least three chromosomes in this group show with less intensity than the others.

In certain chromosome plates, there may be a kind of gradient in the optical retardation from the chromosomes at the center of the plate out to those at the edge of the c-metaphase group. Some plates certainly show the chromosomes at the periphery of the group, or some groups of chromosomes at the periphery, to be differently retarding from the other chromosomes (Figs. 56, 57, 60, 63). In many groups there is only the slightest indication of this, and no indication at all in others. Whether this is due to some kind of artefact, such as perhaps a piling up of the cytoplasm in this part of the cell, must be looked into. As taken up in the discussion, there are some reasons for believing this could be a true biological phenomenon.

With the exception of the terminal variations in optical retardation,

Figs. 62—70. Chromosomes of the Landschütz mouse ascites tumors, hypertetraploid strain, at colchicine metaphase. Sodium citrate pre-treatment, fixation and squashing in 60 % acetic acid, and hardening in formalin. Mounted in distilled water. All photographed with 100× shearing objective except Fig. 62, which was photographed with 100× double-focus objective. In some cases the ghost image was cut off and is not shown here. In Fig. 64 the ghost and real images are divided by heavy line. — Fig. 62. One of the metaphase groups in which the chromosomes show a general uniformity in their optical paths. Several of them, however, are not properly oriented. Some of the chromosomes show a weaker retardation through the centromeric end than through the rest of the chromosome. Others are equally retarding from end to end. At the arrow is one of the atypical, metacentric B chromosomes with a relatively weak retardation through the center of its length. Fig. 63. The chromosomes at the arrows are the atypical, secondarily-constricted A chromosomes. The area distal to the secondary constriction is differently retarding from the rest of the chromosomes. Fig. 64. The two chromosomes at the point of the arrow especially stand out as differing in mass from most of the others. The atypical, metacentric chromosome shows a retardation through both halves of one arm quite different from that through the other arm. Along side of it, is a chromosome with a quite different optical path all along its length except at the point of the centromere. At the top of the photomicrograph there is one, and at the left side of the plate another chromosome, with different optical paths from the remainder of the chromosomes. In the center of the left edge of the plate is another of the metacentric chromosomes showing a variant retardation along most of its length. A line separates the ghost image from the real image. Fig. 65. At the arrow is a chromosome with two knob-like structures having a different optical path from the rest of the chromosome. The uppermost chromosome in the plate differs in optical path from the rest of the chromosome group. Fig. 66. The chromosome at the arrow has a chromomere-like structure on each chromatid, which varies in optical path from the rest of the chromatin, the centromere excepted. Fig. 67. One arm of the metacentric B chromosome is somewhat shorter than the other, and displays a greater optical path. Fig. 68. Metacentric B chromosome. One arm is more highly retarding at its end. The other arm has a retardation all along its length equivalent to the ends of the other arm. Fig. 69. Again one arm of the metacentric B chromosome with a greater retardation than its other arm. Fig. 70. The secondarily-constricted A chromosomes. The region from the point of its secondary constriction out to the distal end is more highly retarding than the rest of the chromosome.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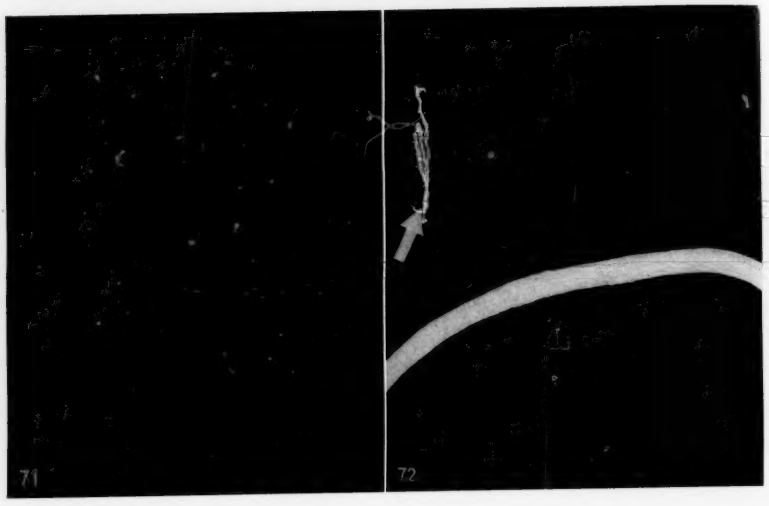
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the chromosomes in general show a uniform optical path all along their lengths. However, there is some evidence for believing that the abnormal, secondarily-constricted A chromosome, and the abnormal, metacentric B chromosome show a more exaggerated, though probably similar optical variability, than do the other chromosomes. These two chromosome types are typical for the Landschütz tumor (TJIO and LEVAN, 1954). The abnormal A often displayed a greater retardation from the point of its secondary constriction out to its distal end, as seen in the two A chromosomes at the arrow in Fig. 63. Fig. 70 illustrates the same phenomenon. The terminal regions of the abnormal, metacentric B chromosome were sometimes more highly retarding for a considerable distance along the chromosome, relative to the more weakly retarding central length of the chromosome. There seemed to be a tendency for one arm of the chromosome to retard the light to a greater degree further along its length than the other arm of the same chromosome (Figs. 80, 83—85). These optical differences in the A and B chromosomes occur in metaphase groups in which other chromosomes are in general at a stage when they are uniformly retarding the light along the whole chromosome arm. Interesting and possibly as important as such differences could be when displayed by rearranged chromosomes, it is essential to be cautious here, since in such an introductory study as this, so much more attention is easily focused on chromosomes otherwise known to be abnormal.

The chromosome at the arrow in Fig. 65 has a chromomeric-like differentiated segment in both chromatids a little more than a third of the distance from its end. In Fig. 66 the chromosome at the arrow displays a chromomere-like body on each chromatid at about the center of the chromosome.

In comparing the several chromosomes of any one cell, it is to be remembered, that with the 100× sh objective, only about half of the image, and consequently about half of the total number of chromo-

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Figs. 71—72. Chromosomes of the Landschütz mouse ascites humors, hypertetraploid strain, after treatment with a heavy metal to increase density. Colchicine metaphase. Sodium citrate pre-treatment, fixation and squashing in 60 % acetic acid. Chromosomes of Fig. 71 were treated for four hours in a saturated solution of phosphotungstic acid (general protein "stain"). Chromosomes of Fig. 72 were treated for four hours in saturated ferric alum of 2 % lanthanum chloride (chromatin "stain"). Mounted in distilled water. 100× shearing objective. — Fig. 71. Note the optically differentiated ends of most of the chromosomes. Ghost image lies across the bottom third of the plate. Fig. 72. The chromosome at the arrow has a different retardation from the others of this group. The line separates the ghost from the real image.

somes, are available for study. This is due to the overlap of the ghost image onto the real image. Such images must in addition be studied with the lower power objectives to determine relative retardations of the other half of the group. Unfortunately, also to be disregarded are all the chromosomes not lying flat, except in determinations of total mass. All classes of optical variations described between the chromosomes, and along the length of the single chromosome occur up to the order of 0.1 of a wavelength (546 mμ green line) difference from the chromosome, or portion of chromosome serving as the source of comparison.

3. Discussion of the interference images of the fixed chromosomes

A. *Behavior of chromosomes established by other methods likely to be related to the observations in interference contrast*

Since the chromosomes of the Landschütz ascites tumors studied here were treated with colchicine, it is not possible to separate completely their colchicine-effected behavior from their normal behavior. Obviously chromosomes of untreated cells must be studied. The asynchronous variability observed here for the terminal chromosomal regions might then be partially an effect of the colchicine treatment, a normal condition, or a condition perhaps exaggerated by the malignant state of these cells. That colchicine does affect the synchrony of the chromosome cycle is very likely, as will be further discussed below. Colchicine is well known to cause changes in chromosome morphology. The chromosomes of these mouse ascites tumors were reported to be more contracted, and the spindle regions more stretched out than in untreated cells (TJIO and LEVAN, 1954). Every chromosomal modification induced by colchicine was found to be present in untreated tumors, but more rarely.

It was noted by the same workers that, for the Landschütz tumor, orcein-stained metaphase chromosomes have a negative heteropyknosis at their proximal ends. On the other hand, during telophase the centromeric regions are the chromosomal sectors with the highest degree of positive heteropyknosis. In these preparations, the chromosomes tapered off at their distal ends.

Upon pretreatment with certain heavy metals, LEVAN (1946) demonstrated a heterochromatin at the centromeric regions in *Allium* chromosomes at metaphase (root tip cells). At other stages these chromosomes show a heterochromaty in this region, but only with the pretreatment

was it possible to demonstrate it during the metaphase and anaphase stages of the same cell.

ÖSTERGREN (1950) mentions as an example of changes in the state of pyknosis that the kinetochore regions are negatively heteropyknotic during metaphase of mitosis, but points out that they often are isopyknotic at the first meiotic division. It is well known, also, that chromosomal regions which are represented as heterochromatic knobs at interphase of cell division may correspond to secondary constrictions at metaphase. For a review of the literature on the function of the centromere, see LIMA-DE-FARIA (1958).

The phenomena indicated are then similar to those observed for the chromosomes of the mouse ascites tumors with the interference microscope. This cyclic variability in the optical retardation of the terminal chromosomal regions ought to be studied in relation to the total chromosomal mass.

The whole chromosomes exhibiting a differential light retardation may be specific chromosomes, or, by chance, any number of different chromosomes in the various cells observed. Sex chromosomes are known to be heteropyknotic at different stages of the division cycle in some tissues of many species studied. The sex of the individual in which the Landschütz strain of tumor originated is unknown. TJIO and LEVAN (1954) considered the possibility that a chromosome in this tumor, which sometimes appeared negatively heteropyknotic is possibly the Y chromosome, since the Y is known to be negatively heteropyknotic during the contraction stage of some species, as for example, in the grasshopper. Although the Y chromosome in the mouse has no differential behavior in many mitoses, it was considered that, in these tumors, it might be in a physiological environment favoring heteropyknosis. In insects there are cases known in which the chromosomes are heteropyknotic in one sex only. In plants, regions which are positively heteropyknotic during the resting stage often have quite the same appearance as other chromosomes at metaphase. The supernumerary chromosomes in *Anthoxanthum aristatum* are positively heteropyknotic during the resting stage, and negatively so at metaphase. In numerous other species, the reverse is true of these chromosomes (MÜNTZING, 1958; FRÖST, 1959 a, 1959 b). As pointed out by ÖSTERGREN (1950), such chromosomes that change their character of heteropyknosis should go through a stage when they are isopyknotic. In a study of the amphidiploids of *Nicotiana*, KOSTOFF (1939) found that the chromosomes of one parent species were bigger and more deeply stained than the chro-

mosomes of the other parent. In the F_1 hybrids, some univalents showed a weaker intensity of stain. In the amphidiploids some bivalents were quite normally stained, while other bivalents were only very faintly stained, and almost transparent. In the faintly stained bivalents, the spindle regions were often intensely stained.

OHNO *et al.* (1958), maintain that, in the spermatogonial mitosis of the rat, the small Y chromosome stands out in prophase because of its positive heteropyknosis. The large X chromosome is isopyknotic. At metaphase I, the X is long, slender, and easily recognized. By this time, both the X and Y are isopyknotic to the autosomes which have attained a high degree of contraction. Almost without exception, at anaphase a positive allocycly of the X and Y is observed. At interphase, the X and Y remain heavily condensed, while the autosomes become fine, thread-like structures. At metaphase II, the X chromosome still stands out by virtue of its deep staining. Upon the completion of the second meiotic division, the autosomes begin to lengthen, but the sex chromosomes remain greatly contracted. The same workers reported, for the rat liver cells, that neither the X nor the Y in the male demonstrated a positive heteropyknosis. Furthermore, only one of the two X chromosomes in the female was observed to be heteropyknotic (OHNO *et al.*, 1959).

Then heterochromatin need not necessarily demonstrate heteropyknosis. Considering the variable behavior of the sex chromosomes, it is not justified to assume that a chromosome displaying a different optical retardation from the others is necessarily a sex chromosome, or that it is heterochromatic, though this remains a possibility.

Recently, an asynchronous behavior has been reported for the DNA synthesis cycle of heterochromatin and euchromatin (LIMA-DE-FARIA, 1959). There is a differential DNA-labelling sequence in the two X chromosomes of the Chinese hamster (TAYLOR, 1960), and differences in the morphology of the two chromosomes (YERGANIAN *et al.*, 1960).

There is some evidence that colchicine effects a different degree of coiling in various chromosomes of the same cell. Recently, SASAKI (1960) reported that the larger somatic chromosomes of the tissue cultures of the rat, golden hamster, and man contract more strongly upon colchicine treatment than do the shorter ones in the same cell. Dry mass measurements would be more sensitive in detecting such differential behavior among the chromosomes than is measurement of chromosome length.

BAJER (1959) found differences of up to 16 per cent in the length of homologous chromosomes of endosperm cells. The supernumerary B

chromosomes in maize show a distinct premature shortening (LONGLEY, 1941). WICKBOM (1949) found in three different species studied, *Bufo bufo*, *Salamandra salamandra* and *Hyla arborea*, that shortening is proportionately much greater in long chromosomes than in small ones for the chromosomes between mid-prophase and metaphase. Between early prophase and metaphase, on the other hand, all chromosomes are shortened by the same percentage, regardless of length. During early prophase then, shortening is proportinately much greater in the shorter than in the larger chromosomes. Contrary to this, BAJER has found, for living endosperm cells, that all the chromosomes shorten in the same way at all the stages, quite independent of their lengths (1959). YERGANIAN and his co-workers (unpublished) find a differential contraction among the chromosomes of tissue cultures of the Chinese hamster upon colchicine treatment. These are easily observed due to the wide range of length of the chromosomes of the hamster, which makes possible recognition of the same chromosome pair from one cell to another.

It is not known how much such differential contraction of the chromosomes, if any, is a cause of the variations in optical retardation detected among the chromosomes of the Landschütz mouse ascites tumors. Contradictory results in the literature on changes in chromosomal length indicate that such a phenomenon is somewhat species-dependent and tissue-dependent. It must also be affected by environmental and experimental conditions, as well as by the physiology of the cell. Such differences are of interest in themselves, but this presents a background of variability against which any differences due to variables in concentration of substances in the chromosome would have to be measured.

Changes in the length and volume of the mitotic chromosomes of living plant endosperm cells were reported by BAJER (1959). Chromosomes decrease rapidly in length until the dissolution of the nuclear membrane, and then continue to decrease in length until metaphase. In metaphase there may possibly be a short period when the chromosomes do not change their length. They start to decrease in length again just before the onset of anaphase, and continue to decrease throughout anaphase. Especially significant for an interpretation of the interference patterns of the mouse ascites tumors, is BAJER's observations that in the same cell, homologous chromosomes can have slightly different lengths, and that the course of chromosomal changes is not the same for all the homologues.

The volume of the chromosomes of the living endosperm cells increases until late metaphase, when it reaches a maximum. Volume then

begins to drop before the start of anaphase, and continues to decrease throughout the anaphase. Whether these changes in volume are due to variations in content of water, or to synthesis and loss of other material is not known. Such phenomena must also have significance in interpreting optical variations among the chromosomes of the Landschütz ascites tumors.

The differences in the optical retardations of the chromosomes situated on the periphery of the chromosome plate, as observed in a few cells, could be due to a differential behavior of the chromosomes dependent upon their proximity to the cytoplasm and to the presence of certain cytoplasmic enzymes. This would be followed by a kind of preferential arrangement of the chromosomes on the metaphase plate. Some of this orientation is then kept through the colchicine metaphase, and is sometimes still expressed after squashing, as the arrangement seen in these photomicrographs. The possibility that it is an artifact created during the fixation process, or during the pre-treatment with sodium citrate cannot be excluded without further work.

In colchicine-telophase of living endosperm cells, structural changes in the chromosomes seem to begin in almost all cells from the periphery of the chromosome group, then proceed toward the center (MOLÈ-BAJER, 1958). It was very often observed that the chromosomes at the edges of the nucleus were changed very considerably, while the chromosomes at the center retained their original appearance.

The observed differences in the optical retardation of the abnormal A and B chromosomes typical of the Landschütz mouse ascites tumor could be a result simply of the rearrangement of these chromosomes. Another possibility is that they contain portions of the sex chromatin, which is certainly allocyclic in its behavior in several normal tissues and stages of the division cycle. In the Ehrlich ascites tumor, to which this Landschütz strain is very likely related, the A chromosome was reported to have difficulties at the anaphasic separation (TJIO and LEVAN, 1954).

B. *Interferometry in studies of the chromosome, and problems in measuring at chromosome level*

The term "heteropyknosis" has been used here to signify that a chromosome or chromosomal segment differs from the majority of the chromosomes of the same cell in its apparent intensity of stain. As pointed out by ÖSTERGREN (1950), a chromosome is heteropyknotic

either because it is thicker or thinner, as a result of a differential coiling of the chromosome threads, or because of a difference in concentration of material in the chromosome. That several different kinds of heterochromatin exist with either specialized or genaralized functions is suggested by KAUFMANN (1960). Heterochromatin behaves quite differently, depending upon the tissue as well as upon the various stages of the life cycle of the cell. There may be, at least in some instances, some simple relation between the state of pyknosis based on staining affinity and the relative degree of optical retardation. Some of the confusion can be partially resolved by the interferometric determination of the thickness of what should be heterochromatin, and comparing this with the relative thickness of the euchromatic chromosomal material. For such determinations the chromosomes can be regarded as isolated, since in well-prepared squashes, the cytoplasm lying above and below the chromosome must be negligible.

Some chromosome fixation ought to be coupled with specific extraction procedures to reveal information about the quantities of the several chromosomal components in the comparative sense. Extraction procedures have been used in conjunction with specific staining of the chromosomes (KAUFMANN, 1960, for review). A preliminary report on fixation and extraction in interferometric technique has been made by DAVIES, DEELEY and DENBY (1957). By degrading the heterochromatin and euchromatin of the same set of chromosomes we should be able to determine the relative proportions of protein, desoxyribonucleic acid and ribonucleic acid.

Whether the total chromosomal masses or the mass of individual chromosomes varies from tissue to tissue, as seen over different stages of the life cycle of the same cell, is not known. Now that the content of total desoxyribonucleic acid has been extensively reported for different tissues, both normal and malignant, attention could well be turned toward the prospect of quantitating individual chromosomes. MELLORS *et al.* (1954) report, on the basis of their interferometric studies on the germinal cells of the mouse, that there is an increase in the concentration of organic mass as well as nucleic acid in the prophase chromosomes. This would indicate the nucleic acids need not be the only variable which could contribute to differences in the optical paths of the chromosomal material. Little is actually known concerning the behavior of the classically-defined heterochromatin, nucleolus-organizers, and secondary constrictions of the chromosomes of different tissues. Aneuploidy even for a single chromosome in a diploid tissue could well

bring with it all categories of subtle effects on the physiology of the entire karyotype.

When chromosomes are measured individually, differences, though small on the basis of the quantity of material present in the entire complement of chromosomes, should be detectable and measurable in many cases. This has been well demonstrated by STICH and NAYLOR (1958), who measured the variation of the DNA content of single chromosomal segments in the salivaries of *Chironomidae* with the visible light microspectrophotometer, following staining with Feulgen. An eight-fold increase in the DNA content of puffed regions was found before and after the accumulation of the DNA-positive globule. The increase could not be attributed to alterations in the degree of polyteny. The variation, though representing an eight-fold increase, still amounts to only 10 per cent of the DNA content of the whole chromosome. That the same chromosomal complement can behave differently in different tissues, so as to cause changes in even the gross chromosomal morphology is seen from the work of BEERMAN (1956). It has been shown that the chromosomes in the developing larvae of certain insects have puffs in different regions correlated with different areas of the embryo.

All classes of chromosomal variations described here can be differing in optical path from the remainder of the chromosomal material in the cell up to the order of at least 0.1 of a wavelength at the 546 mμ green line. With the special half-shade eyepiece used with the interferometer, differences should be measurable down to the order of 0.005 of a wavelength. With the Davies-Deeley integrator (DAVIES and DEELEY, 1956) the mass of an isolated object can be measured to the accuracy of 0.001 wavelengths. Chromosomes of the same cell might be measured against their cytoplasm, or the chromosomes simply regarded as isolated in those squashes where the cytoplasm is spread so thin as to have almost a negligible retardation, relative to that of the chromosome. The object need not have a regular outline.

Some chromosomes and segments of chromosomes may be too small for accurate measurement of their retardations by the unaided eye. Should this prove to be the case, the trace displacement method might be used (HALE, 1958). The chromosomes are photographed with interference fringes in the field of view, and intensity measured from the film with a densitometer (accuracy, 0.0005 λ). Areas down to the limit of the resolving power of the microscope can be measured in this manner. Also the multiple beam interference microscope, and corresponding biological methods devised by MELLORS *et al.* (1954) might prove valuable.

The interferometric method is still wrought with numerous technological problems, and much large-scale and very fine work on the chromosomes must await improvement and simplification of measuring procedures, and perhaps instrumentation. There is encouragement from the fact that, at the metaphase stage alone, there are differences in the optical paths of the chromosomes feasible of measurement with present techniques, even though this is now a painstaking work. The metaphase stage is a time of the division cycle that more classic work has led us to think of the chromosomes as generally being in a rather static status, all of them synchronized with one another, with the exception of the sex chromosomes, in some instances. As seen above, the chromosomal variabilities viewed interferometrically seem related to phenomena detected by staining and light microscopic methods, and in living cells studied with the phase microscope.

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SUMMARY

An introductory study has been made to the immersion interferometry of the Landschütz mouse ascites tumor and its chromosomes. In such microscopy, each cell type presents its own array of methodological problems, and problems in image interpretation, and these are discussed here in relation to this cell. For study of the intact cell, living tumors were used. There is a description of suitable immersing media and the concentrations found most desirable for definition of the dividing and non-dividing cell. For study of the chromosomes, suitable fixation was combined with proper handling and mounting to insure maximum contrast and sensitivity. A series of photomicrographs illustrates

the kind of image in which determinations of quantity can be carefully related to cellular detail and behavior, and to chromosomal morphology.

A survey study is made of the various interference-contrast images of the non-fixed cell over its life cycle. Similarities and differences in optical path among the various chromosomes of metaphase cells, and along the lengths of the chromosomes are described. These are all discussed in terms of related phenomena observed in other cell types by more established forms of microscopy. The meaningfulness of the instrumental limits of accuracy, when applied to such a cell as the mouse ascites tumor, is considered in relation to current problems of cytology and cytogenetics.

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INITIATION OF DNA SYNTHESIS AT SPECIFIC SEGMENTS IN THE MEIOTIC CHROMOSOMES OF MELANOPLUS

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INTRODUCTION

THE study of DNA synthesis at meiosis is of particular significance in connection with crossing-over.

The chromosomes of most organisms attain their largest size at the prophase of meiosis. At this stage, for example, in *Ornithogalum virens* they may become as long as 110 microns (LIMA-DE-FARIA, SARVELLA and MORRIS, 1959). Tritium beta particles have an average range in tissue of 1 to 2 microns (HUGHES *et al.*, 1958). With the use of tritiated thymidine one can then study DNA synthesis along the meiotic chromosomes with a degree of detail not attainable in the much shorter mitotic chromosomes.

In previous works (LIMA-DE-FARIA, 1959 a and b) the incorporation of tritiated thymidine into the meiotic chromosomes of the grasshopper *Melanoplus differentialis* was described. This study was made mainly in the zygotene and early pachytene nuclei. At these stages, owing to the *bouquet*, the chromosomes are closely packed together, but at late pachytene the autosomes become free from each other and the largest chromosomes of the complement attain a length of 30—40 microns. This allows a study to be made of the replication process along the DNA chain at a time when the chromosome is most extended and most easily recognizable. The present work deals with autoradiographic and spectrophotometric studies made at this later stage.

MATERIAL AND METHODS

A. *Autoradiography.* — Grasshoppers of the species *Melanoplus differentialis differentialis* (2n=22+X in the male) were injected with tritiated thymidine at the last nymphal stage. At this period of develop-

ment the testis shows many spermatocytes in the onset of meiosis. Tritiated thymidine, purchased from Schwarz Laboratories, Mount Vernon, N.Y., was injected into the abdominal cavity at a concentration of 500 μc/ml (specific activity 0.360 curie/mM). Each animal received 0.02 to 0.04 ml. The animals were killed and the testes immediately fixed in acetic-alcohol 1:4. This was done 9 to 12 days after injection, the period of time necessary to obtain late pachytene with the tritium label.

Both squashes and sections of the testis were stained according to the Feulgen technique as described in LIMA-DE-FARIA (1959 b). To insure intimate contact of the stripping film emulsion with the cells care was taken to see that the cells were evenly distributed and well flattened when squashed. For the same purpose the sections were cut with a rotary microtome at a thickness of 2 microns. Autoradiographic stripping film from Kodak, Ltd., England (AR-10) was applied in the usual way. All the squash preparations were made the same day and exposed for 23 days in boxes kept in a refrigerator. The sections were exposed for a period of 2 to 3 weeks. Comparison of cysts with different types of labelling was only made within the same slide where the exposure time was the same for all spermatocytes of a testis. Other details of the technique are the same as described in LIMA-DE-FARIA (1959 b). Colchicine was not used in any of the experiments.

B. *Photometric measurements.* — A microspectrophotometer designed by CHANCE, PERRY, ÅKERMAN and THORELL (1959) available at the Department of Pathology, Karolinska Institutet, Stockholm, was used for this purpose. A description of the apparatus and a discussion of its applications is also found in THORELL (1960). This is a highly sensitive automatic recording microspectrophotometer capable of measuring small light absorptions in localized cell structures with a high degree of accuracy.

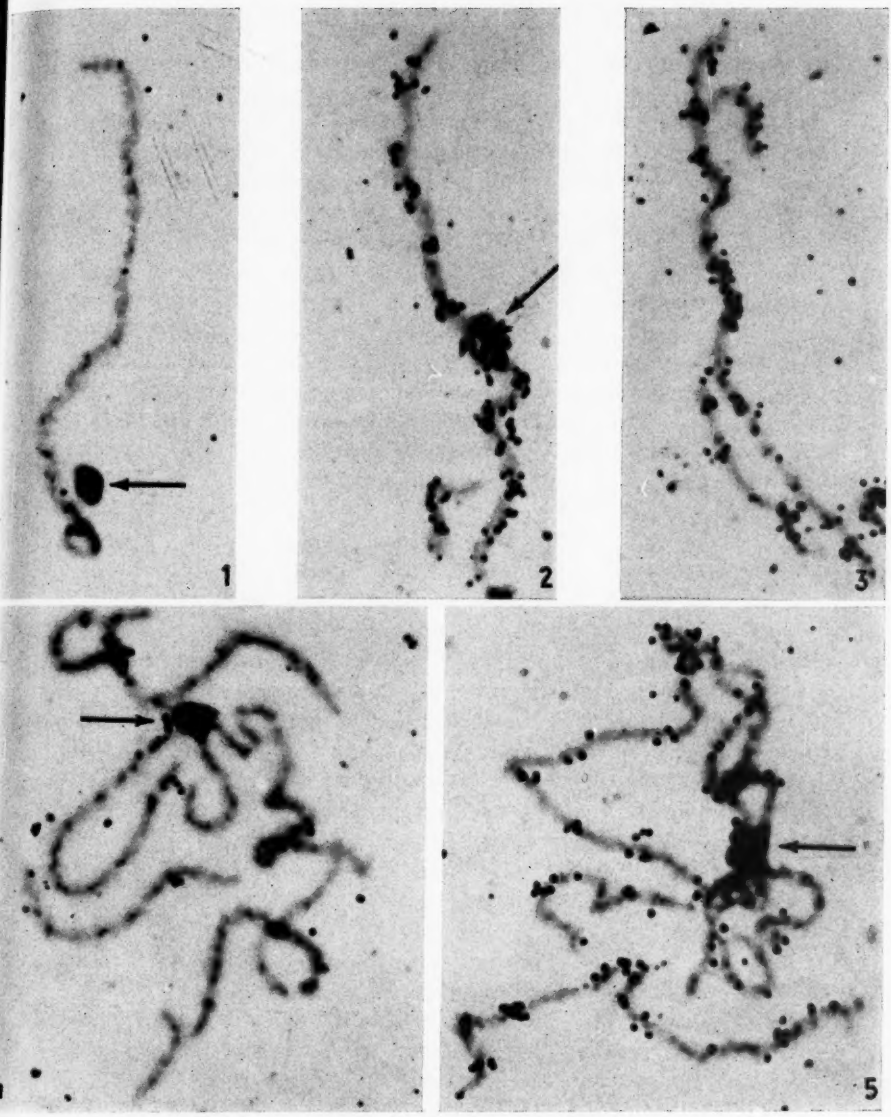
The microspectrophotometer has been used originally to detect respiratory enzymes in a portion of the living cell less than one micron in diameter and in the amount of 1×10^{-18} mole at a signal-to-noise ratio of $\sim 50:1$. This noise level corresponds roughly to 2×10^{-20} mole or $\sim 10^4$ molecules of the substance under study. The apparatus consists mainly of the following parts. A tungsten light source and a grating monochromator illuminate a microscope containing Grey-polaroid reflecting-refracting optics. This light is then reflected with a 45° mirror into a system of vibrating mirrors which move in a vertical direction and flash the light from two positions of the image intermittently upon an adjustable aperture. An S-4 photo tube and an amplifying circuit transmit the

signal to a recorder which plots the difference between the "reference" and the "measure" signals, since this reference is almost equal to the optical density difference. This means that when locating in the microscope the chromosome that one wants to measure there should be no other chromosome in the region where the "reference" beam is located. The scanned area is controlled directly by the eye at the plane of the photocell aperture, and the reproducibility of the absorption measurements is less than 1 % using a time constant of 2.5 seconds.

The same preparations which were used for the autoradiographic studies were employed for the photometric measurements. Cargille's immersion oil $N_D=1.5150$ was applied over the autoradiographic film and a cover slip placed on the top. A drop of glycerin was then placed over the cover slip, since this is the liquid employed for the immersion objective. The wave length used was 560 mμ. The optics of the microscope consisted of an objective ultra-fluar, Zeiss, 100× N.A. 0.85, an ultra-fluar condensor (Zeiss) N.A. 0.80 and a quartz eyepiece 3.5×. Chromosomes were selected which were completely flattened and well stretched out. Furthermore, only chromosomes which were oriented in a plane parallel to the long axis of the preparation were used, since those which have other orientations are much more difficult to center segment by segment on the microscope stage due to the very small aperture of the photocell.

The chromosome under study was divided into 1.3 micron segments by means of an eyepiece graticule. The chromosome segment to be measured was centered in the microscope, and then at the plane of the photocell. The photocell aperture corresponds to an area at the plane of the object equal to 0.7×0.7 microns. Once the absorption of this segment was registered the preparation was moved, so that a new chromo-

Plate 1. Fig. 1. Unlabelled pachytene autosome and heteropycnotic sex chromosome (arrow). Fig. 2. The sex chromosome (arrow) is in contact with two autosomes, one in the upper half of the figure, the other in the lower half. In the upper chromosome clusters of silver grains are distributed at regular intervals. The DNA synthesis is being initiated in this chromosome. In the lower chromosome the synthesis has progressed a little further, as evidenced by the presence of silver grains filling the spaces between the clusters. The few grains seen over the sex chromosome are due to the overlap of this autosome and the sex chromosome. Fig. 3. Another case similar to the chromosome in the lower half of Fig. 2. The presence of silver grains between the clusters indicates that the synthesis has progressed further. Fig. 4. Unlabelled spermatocyte nucleus at late pachytene showing the 11 autosome bivalents and the X chromosome (arrow). Fig. 5. Spermatocyte nucleus at late pachytene showing the sex chromosome unlabelled (arrow) and the autosomes with clusters of silver grains distributed at regular intervals.



Plates 1—4. ***Melanoplus differentialis.*** **Spermatocyte chromosomes at late pachytene. Feulgen stain. — ×2000.**

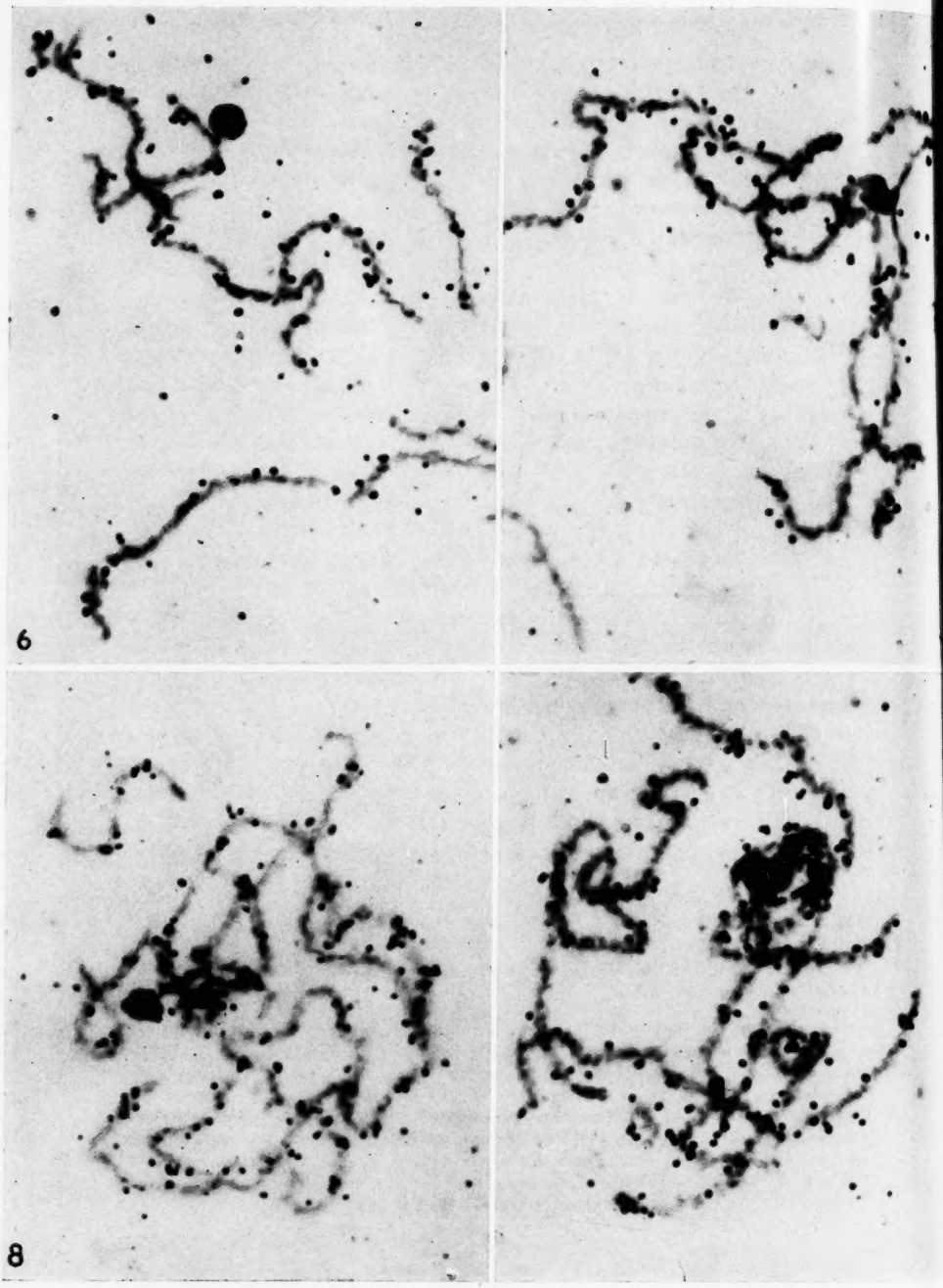


Plate 2. Figs. 6 to 9. Nuclei at the initiation of DNA synthesis. The sex chromosome is not labelled while the autosomes show clusters of silver grains distributed at regular intervals.

some segment was centered and measured. This procedure was repeated until the whole chromosome was measured.

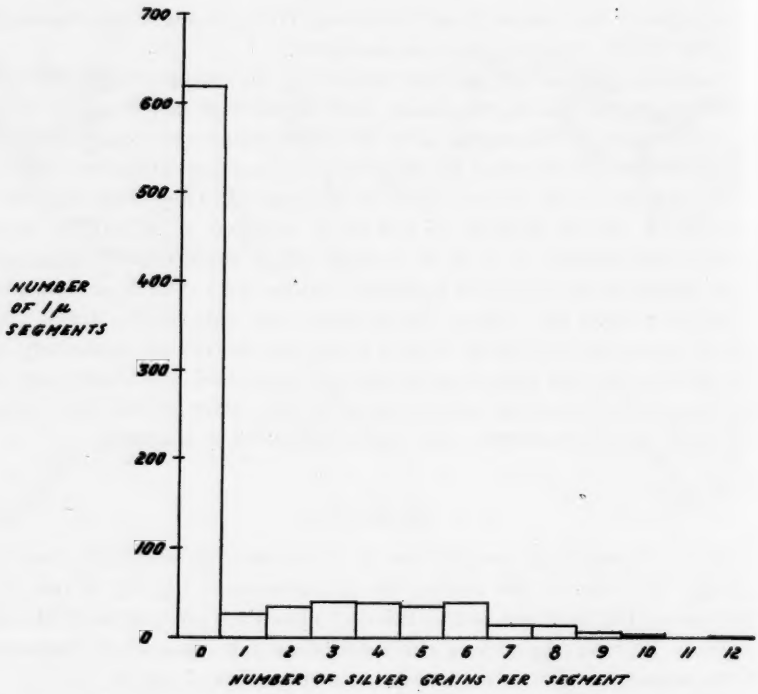
Absorption measurements were made: (1) on chromosomes without silver grains, (2) on chromosomes with clusters of silver grains, and (3) on the same chromosomes after the silver grains were removed from the emulsion by employing the technique of GALL and JOHNSON (1960). The slides were first cleaned from immersion oil. They were then immersed for 10—15 seconds in a 0.75 % solution of $K_3Fe(CN)_6$, and treated subsequently by a 20 % $Na_2S_2O_3$ 5 H_2O solution for 3 minutes. After that they were washed in distilled water for 1 minute, and stayed in running water for 1 hour. The preparations were finally dried. The use of the eyepiece graticule makes it possible to relocate accurately in the microscope any chromosome segment measured previously and to determine once more its absorption after the silver grains have been removed. All chromosomes were again measured in this way.

RESULTS

In the chromosome complement of *Melanoplus differentialis* there is a large variation in size among the 11 autosomes (Fig. 4). A few are quite small but most are large, attaining a length at pachytene of 30—40 microns. Both short and long chromosomes can be completely flattened if the squashes are carefully made, as seen in Figs. 1 and 4.

When analyzing the labelling in the autosomes one would expect to find at the beginning of the synthesis a few silver grains evenly distributed along the entire chromosome. The number of grains would then successively increase until full synthesis was reached. Subsequently the labelling would start tapering off until it finally disappeared. Instead of this picture of regularly distributed label one finds at the onset of synthesis that the silver grains appear in clusters distributed along the chromosome (Figs. 5—9).

It was found previously that the sex chromosome synthesizes later than the autosomes (LIMA-DE-FARIA, 1959 a and b). This information was obtained in sections of the testis. The spermatocytes are grouped in cysts which are synchronized in their meiotic stage and in their DNA synthesis. These cysts are formed at the base of the testicular tubule and are successively pushed forward during the process that leads to the formation of spermatozoa. Cysts that contained spermatocytes synthesizing only in the sex chromosome were found to be at later stages than cysts which were synthesizing only in the euchromatic autosomes.



Text-Fig. 1. Distribution of silver grains along the pachytene chromosomes.

As a result, the sex chromosome present in each nucleus can be used as a control, giving information as to the stage of synthesis of the autosomes.

The labelling occurring along the chromosome as evidenced by groups of silver grains appears most clearly when the sex chromosome is not labelled in the nucleus, *i.e.*, at the initiation of synthesis.

To determine whether the silver grains actually occurred in groups, camera lucida drawings were made only of chromosomes completely flattened along their entire length. With a map measurer each chromosome was divided into one micron segments and the number of silver grains counted for each segment. In Text-Fig. 1 are assembled the results of the countings from 33 pachytene chromosomes along a length of 888 microns from nuclei which were initiating their DNA synthesis. The number of one micron segments without silver grains is 620. The

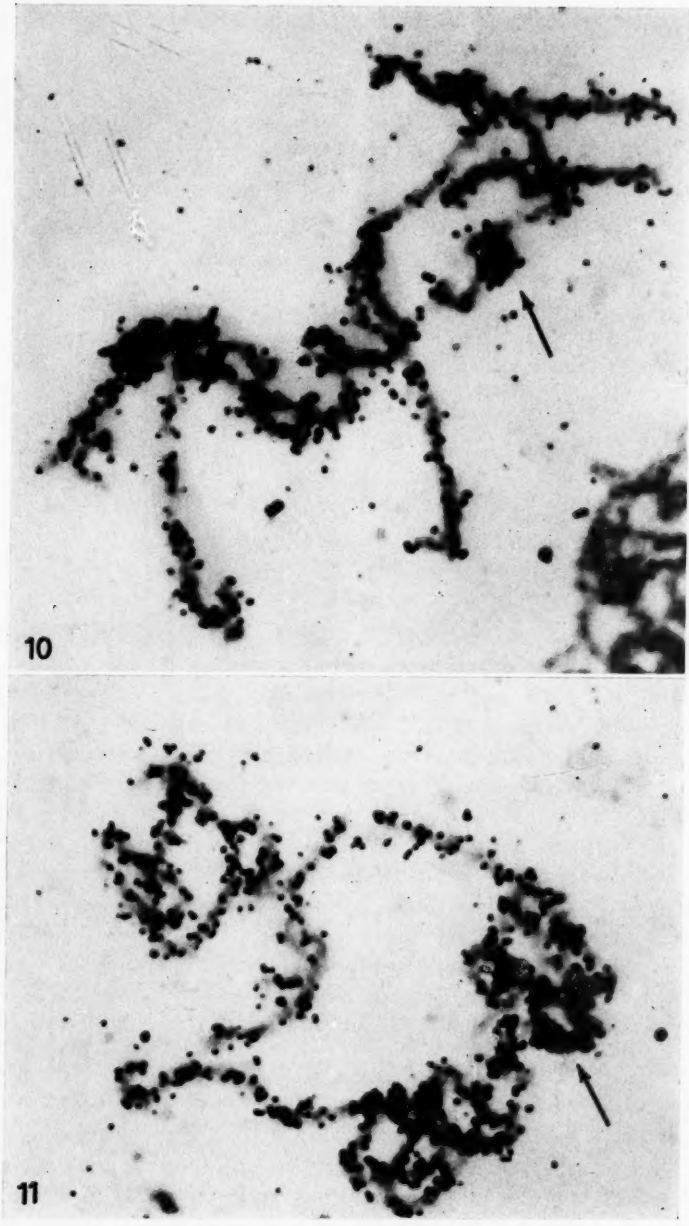


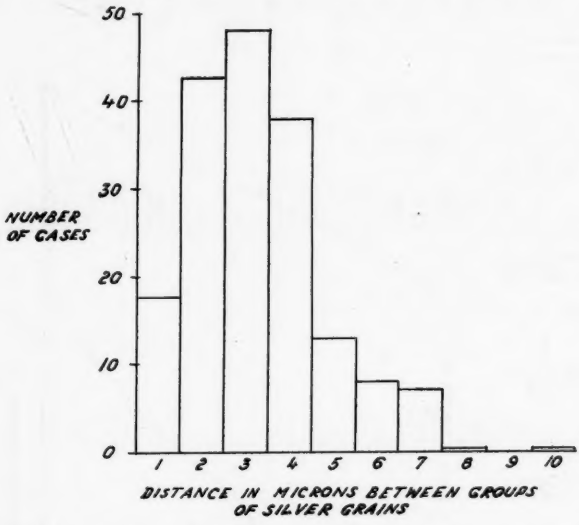
Plate 3. Figs. 10 and 11. Two cells at the height of synthesis showing both the sex chromosome (arrow) and the autosomes heavily labelled.

12

13

14

15

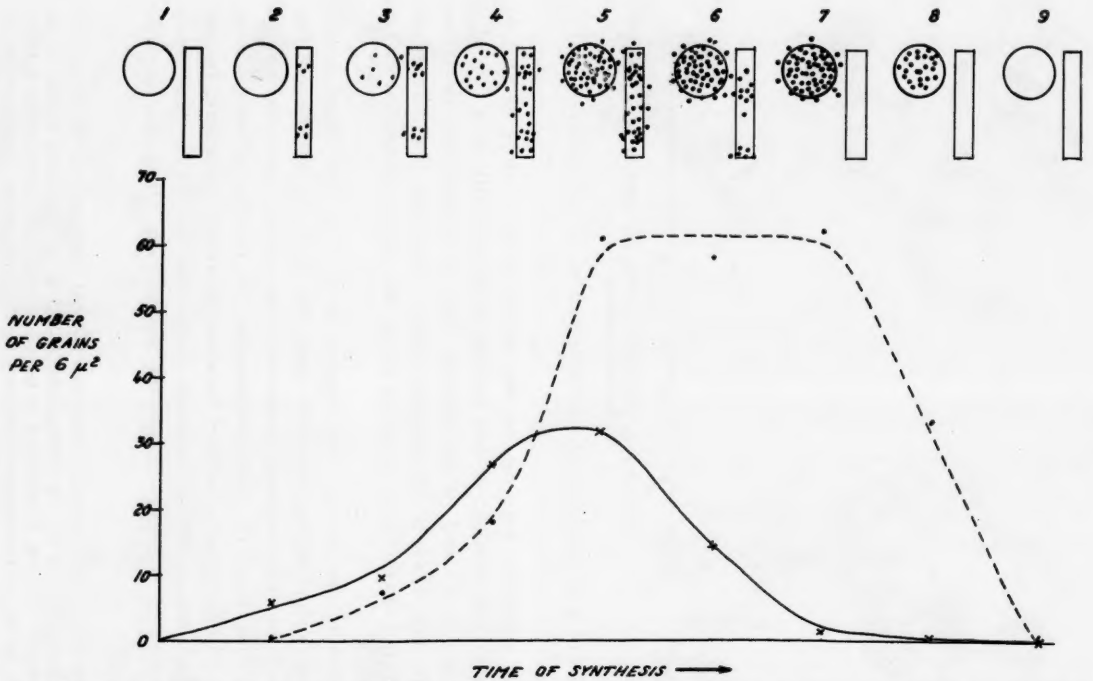


Text-Fig. 2. Distance in microns between groups of silver grains.

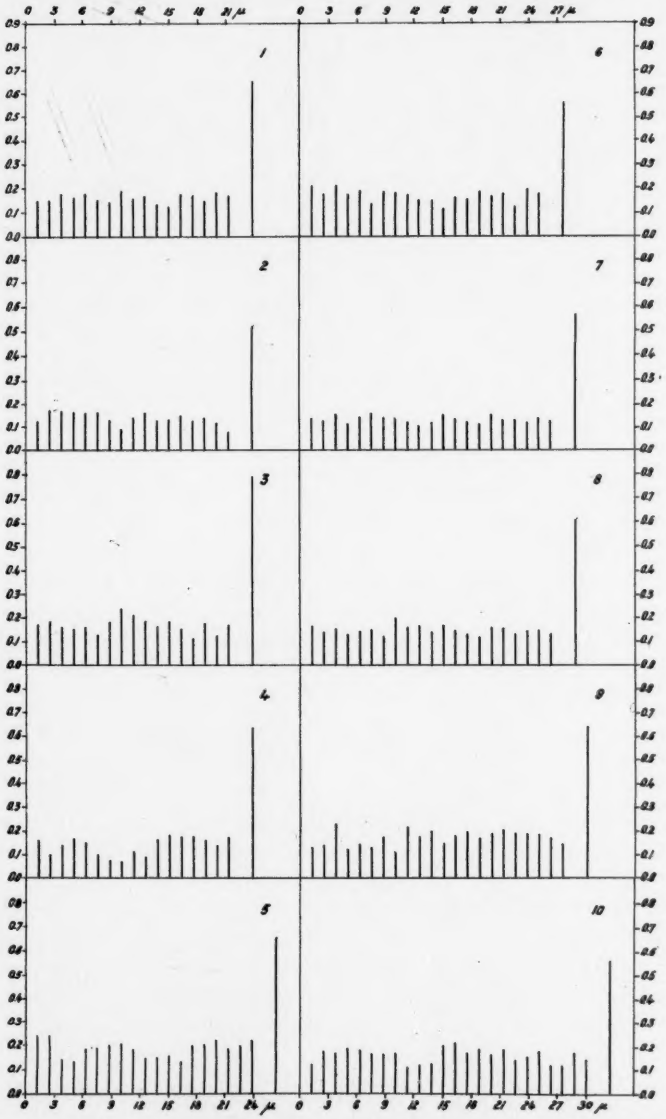
number of segments with one or two silver grains is only 28 and 36 respectively. The number of one micron segments with 3, 4, 5 and 6 grains is 41, 41, 37 and 40 respectively. This is a significant deviation from a normal Poisson distribution curve, revealing that the silver grains are not occurring at random along the chromosome but rather in groups.

The silver grains do not only appear in clusters, but these groups appear to occur at regular intervals along the chromosome. To test this observation the same camera lucida drawings divided into one micron segments were used. In each chromosome the number of one micron segments without grains, present between segments containing two or more grains, was counted. Segments containing one silver grain were only found in 22 segments among the 888 studied. Since one needs to

Plate 4. Figs. 12 to 15. Successive decrease in DNA synthesis in the autosomes, whereas the synthesis of the sex chromosome is still at its maximum. In Fig. 12 the DNA synthesis is declining as indicated by the presence of only a few grains between the clusters. In Fig. 13 the number of clusters has become smaller, and fewer grains are seen between them. Fig. 14. The clusters have now nearly disappeared, their number of grains being reduced to one or two. Finally, in Fig. 15, the synthesis has nearly ended in the autosomes, but is still near maximum in the sex chromosome.



Text-Fig. 3. Sequence of DNA synthesis in *Melanoplus* chromosomes. The large circle represents the sex chromosome, the bar a segment of the autosomes with an area equal to that of the sex chromosome (6 μ^2). There are nine distinguishable periods from the initiation until the end of the DNA synthesis. Dotted line represents the sex chromosomes; full line, the autosomes. The time of synthesis is in arbitrary units.



Text-Fig. 4. Graphic representation of the extinction values of each 1.3 segment of the autosomes and of the sex chromosome (the long bar to the right). Data from Table 1.

TABLE 1. *Extinction values of 1.3 micron segments of Feulgen*

Chromosome	Number of 1.3												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	0.150	0.150	0.183	0.171	0.183	0.156	0.150	0.191	0.162	0.171	0.146	0.128	0.171
2	0.122	0.177	0.171	0.162	0.156	0.156	0.128	0.092	0.146	0.156	0.128	0.128	0.150
3	0.171	0.183	0.171	0.150	0.156	0.128	0.183	0.240	0.212	0.183	0.162	0.177	0.150
4	0.156	0.105	0.140	0.162	0.150	0.105	0.071	0.071	0.110	0.092	0.162	0.183	0.177
5	0.240	0.240	0.146	0.140	0.183	0.183	0.191	0.205	0.183	0.150	0.150	0.156	0.146
6	0.212	0.177	0.205	0.171	0.191	0.146	0.191	0.177	0.171	0.150	0.150	0.116	0.162
7	0.134	0.122	0.150	0.110	0.146	0.156	0.140	0.140	0.122	0.105	0.122	0.150	0.134
8	0.162	0.140	0.150	0.128	0.140	0.146	0.116	0.198	0.150	0.156	0.140	0.156	0.146
9	0.122	0.134	0.225	0.116	0.146	0.122	0.162	0.105	0.212	0.177	0.191	0.150	0.171
10	0.128	0.171	0.162	0.191	0.183	0.162	0.162	0.162	0.110	0.116	0.122	0.205	0.219

account for background radiation half of them were considered as background and half as belonging to a group or constituting a potential cluster. In Text-Fig. 2 are assembled the results of the countings. The groups of silver grains may be spaced as far apart as 10 microns or as close as one micron but there is a distinct peak at 3 microns revealing a regularity of distribution along the chromosome body. This may also be seen in Figs. 5—9. Once the synthesis progresses silver grains begin to appear between the groups (Fig. 3), and finally the whole body becomes heavily labelled (Figs. 10—11).

The sequence of the synthesis was studied in detail. Nuclei belonging to different cysts were selected at random for study of the amount of labelling in both the autosomes and the sex chromosome. The area of the sex chromosome is approximately 6 square microns. Countings of silver grains were made within the same nucleus for both the sex chromosome and for three different segments each having an area of 6 square microns and taken at random among the autosomes. Text-Fig. 3 is based on the average values obtained from silver grain countings made in 54 cells (6 cells for each point of the curve). From the sections it is known that the cells within one cyst are extremely well synchronized so that 5 or 6 cells are quite representative of the 20—30 cells found in a section of a cyst.

As seen in Text-Fig. 3 nine periods of synthesis were distinguished: (1) cells not labelled, (2) cells with only groups of grains in the autosomes, (3) cells with the groups in the autosomes but where the heterochromatic sex chromosome starts to show labelling, (4) cells with

stained autosomes and the sex chromosomes at late pachytene.

micron segments											Mean	Sex chromosome
14	15	16	17	18	19	20	21	22	23	24		
0.171	0.150	0.191	0.177								0.165	0.657
0.128	0.140	0.122	0.078								0.138	0.521
0.110	0.171	0.122	0.162								0.167	0.794
0.177	0.156	0.140	0.177								0.137	0.637
0.205	0.205	0.225	0.183	0.198	0.225						0.187	0.656
0.156	0.183	0.171	0.177	0.122	0.198	0.177					0.170	0.565
0.116	0.105	0.150	0.122	0.122	0.116	0.134	0.122				0.129	0.565
0.122	0.110	0.150	0.150	0.128	0.140	0.140	0.128				0.143	0.618
0.191	0.171	0.183	0.198	0.191	0.191	0.183	0.162	0.140			0.166	0.636
0.171	0.191	0.156	0.191	0.146	0.150	0.183	0.116	0.116	0.171	0.146	0.160	0.552

stronger labelling in the sex chromosome and with the autosomes showing the spaces between the groups filled with silver grains, (5) cells with full labelling in both chromosome types (Figs. 10—11), (6) cells with decreasing labelling in the autosomes and full labelling in the sex chromosome (Figs. 12—13), (7) cells at the end of synthesis in the autosomes but showing the sex chromosome still fully labelled (Figs. 14—15), (8) cells only synthesizing in the sex chromosome, but approaching the end of synthesis, and (9) cells no longer synthesizing in any of the chromosome types.

It may be seen in Text-Fig. 3 that the synthesis starts in the autosomes and slowly increases to a full labelling that reaches approximately 30 silver grains per 6 square microns. The sex chromosome starts synthesizing later, and abruptly increases its labelling until it reaches twice or more than twice the number of grains found in the same area of the euchromatin. Then the synthesis declines in the euchromatin and finally ends. At this time the sex chromosome is still strongly synthesizing until its labelling suddenly vanishes.

Nuclei with the sex chromosome partly labelled at the end or at the beginning of the synthesis were not so common in this material. This indicates that synthesis starts and finishes in this chromosome type in an abrupt way. In the autosomes the synthesis also ends more abruptly than it starts. Groups of grains are less often seen at the end of replication than at the beginning of synthesis.

When the labelling is at its maximum in the autosomes it is observed that not all segments have the same degree of heavy labelling. It is con-

TABLE 2. *Extinction values of 1.3 micron segments of Feulgen stained*

*, grain clusters; ×, part of a cluster or 1 or 2 silver grains. The means of the horizontal
those with grains cannot be taken

Chromosome	Silver grains	Number of 1.3														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Present	× 0.219	0.134	0.156	* 0.374	× 0.281	0.198									
	Absent	0.052	0.105	0.105	0.092	0.162	0.078									
2	Present	0.122	* 0.521	0.140	0.150	× 0.248	* 0.466	× 0.233	0.122	0.110						
	Absent	0.128	0.156	0.146	0.156	0.171	0.128	0.122	0.122	0.105						
3	Present	* 0.298	0.150	0.191	* 0.344	* 0.289	0.099	0.140	* 0.289	0.212						
	Absent	0.198	0.128	0.105	0.082	0.099	0.082	0.110	0.092	0.150						
4	Present	* 0.307	0.071	0.071	* 0.281	0.078	0.082	* 0.354	0.140	0.082	0.082	* 0.219				
	Absent	0.140	0.068	0.071	0.078	0.071	0.056	0.082	0.122	0.082	0.092	0.099				
5	Present	0.140	0.122	* 0.602	0.183	0.205	0.128	* 0.406	0.198	0.171	0.116	* 0.288	0.092			
	Absent	0.116	0.071	0.156	0.128	0.171	0.116	0.140	0.110	0.116	0.092	0.068	0.071			
6	Present	0.150	* 0.364	0.212	* 0.354	0.248	0.273	* 0.467	0.191	0.191	* 0.442	0.225	0.256	0.198		
	Absent	0.150	0.134	0.177	0.171	0.212	0.248	0.177	0.198	0.225	0.198	0.205	0.183	0.162		
7	Present	0.092	* 0.274	0.171	* 0.219	0.092	0.110	* 0.219	0.212	* 0.274	0.082	0.110	* 0.298	0.156	0.128	0.146
	Absent	0.110	0.099	0.146	0.128	0.082	0.140	0.150	0.191	0.156	0.082	0.122	0.171	0.162	0.162	0.219
8	Present	0.116	× 0.205	× 0.212	* 0.248	* 0.385	0.183	0.205	0.198	* 0.256	0.150	0.116	0.191	* 0.326	* 0.256	0.146
	Absent	0.116	0.087	0.092	0.092	0.087	0.134	0.140	0.092	0.110	0.087	0.068	0.128	0.146	0.122	0.087
9	Present	0.171	0.156	* 0.418	× 0.248	0.128	0.191	* 0.374	0.156	0.198	0.171	* 0.299	0.116	0.162	* 0.219	0.140
	Absent	0.162	0.146	0.150	0.177	0.122	0.183	0.162	0.122	0.171	0.140	0.140	0.122	0.116	0.099	0.146
10	Present	* 0.256	0.198	* 0.480	0.146	* 0.418	0.105	0.146	0.110	* 0.219	0.240	* 0.282	× 0.233	0.183	0.191	* 0.324
	Absent	0.219	0.191	0.150	0.219	0.219	0.128	0.134	0.087	0.191	0.212	0.219	0.171	0.156	0.150	0.128

autosomes at late pachytene before and after the silver grains were removed.

column, headed "silver grains present", are only obtained from segments without grains; into consideration for this purpose.

micron segments																Mean
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
																0.162
																0.096
																0.128
																0.131
																0.158
																0.115
																0.087
																0.080
																0.151
																0.110
																0.216
																0.196
*																
0.298	0.110															0.128
0.140	0.105															0.138
		*														
0.110	0.122	0.656	0.171	0.128												0.153
0.105	0.078	0.071	0.116	0.092												0.104
*					×	*										
0.282	0.162	0.219	0.128	0.212	0.282	0.374										0,165
0.140	0.140	0.122	0.099	0.134	0.156	0.198										0.138
		*	*			*				*						
0.183	0.116	0.374	0.335	0.110	0.191	0.344	0.122	0.134	0.087	0.264	0.116	0.191	0.140	0.150	0.128	0.149
0.177	0.092	0.183	0.122	0.122	0.150	0.122	0.105	0.092	0.078	0.078	0.071	0.128	0.140	0.092	0.068	0.130

TABLE 3. *Distribution of extinction values and analysis of variance of* *by the ferricyanide*

Chromosome segments		Extinction									
		0.050	0.060	0.070	0.080	0.090	0.100	0.110	0.120	0.130	0.140
After silver grains were removed	I. Segments which previously had no silver grains	1	3	8	7	8	8	8	14	3	9
	II. Segments which previously had × and ⁂	1	1	3	4	8	0	1	7	1	6
	III. Segments which previously had ⁂	0	1	3	3	7	0	1	6	1	6
Before silver grains were removed	IV. Segments without silver grains	0	0	3	5	4	2	12	11	2	10

values	
0.150	
6	
7	
6	
9	

cluded that the replication of certain segments is already declining when others are attaining full synthesis. It is logical to assume that the groups of grains found at the beginning of the synthesis are present at other loci than are those found at the end of the synthesis. The loci that started replicating first would finish first and those that started last would finish last. There is, however, no way of checking this point. The autosomes of *Melanoplus* at pachytene do not have any cytological markers. The chromomere patterning is not so distinct as that of the pachytene chromosomes of plants and no particularly large chromomeres or knobs are seen. But this is not the same as saying that the clusters cannot be recognized in the same chromosome in different cells. In many cells studied the chromosomes of the complement were found to show the clusters located at corresponding positions.

In microtome sections of the testis, the cells at late pachytene are so well synchronized in their DNA synthesis that all the nuclei of a cyst are usually found at one of the 9 stages represented in Text-Fig. 3. It is to be expected that in the sections the occurrence of silver grains in groups will not be easily detectable at the beginning of synthesis. Although the sections are only two microns thick the chromosomes here are not flattened as in the squashes, and for this reason a less sharp

the various types of regions, before and after removal of silver grains technique.

values													Mean	P
0.150	0.160	0.170	0.180	0.190	0.200	0.210	0.220	0.230	0.240	0.250	0.260	0.270		
6	4	4	2	3	1	4	1	0	1	0	0	0	0.128	I—III $P > 0.2$
7	2	6	1	4	0	3	0	0	0	0	0	0	0.137	I—II $P > 0.2$
6	1	3	1	4	0	3	0	0	0	0	0	0	0.137	
9	2	5	4	14	2	5	1	0	2	1	0	1	0.153	I—IV $P < 0.001$

differentiation into groups is expected. There are, however, cysts in which the phenomenon can be recognized.

The autosomes are not synchronized in their DNA replication. The difference among them is not so extreme as compared with the asynchrony of the sex chromosome, but in the nuclei studied it could be seen that certain autosomes were more advanced than others. In Fig. 2 the sex chromosome (indicated by an arrow) separates two autosomes. The upper one at the beginning of synthesis shows scattered groups of grains, whereas the lower autosome is more advanced, the spaces between the groups being filled with silver grains.

In this grasshopper species the chromomeres are roughly of the same size, and chromomeres which are slightly larger do not seem to occur at regular intervals along the chromosome. This observation strongly suggests that the presence of silver grains in clusters is not related to a localized higher content of DNA. However, to check this point spectrophotometric measurements of the amount of DNA along each chromosome were undertaken.

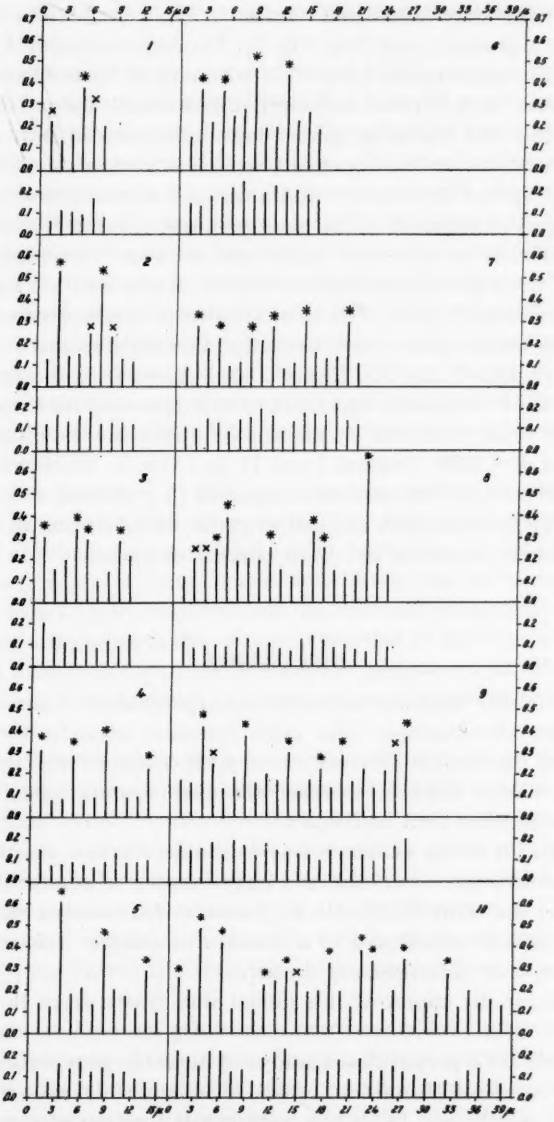
The pachytene chromosomes of *Melanoplus* are 0.9 to 1 micron thick. Each chromosome was divided into 1.3 micron segments and within such a segment a measure of the DNA content was obtained. As the

segment measured should be slightly larger than the aperture of the photocell (0.7×0.7 microns) this results in the center of the area measured being just in the middle of a chromomere or slightly displaced to the periphery, including part of an inter-chromomere space. Repeated measurements of the same segment showed this variation to be only a small fraction of the absorption value.

A first study was made of chromosomes which were free from silver grains to see whether: (1) there was any variation in DNA along the chromosome, and (2) whether this variation occurred at regular intervals. It may be seen from Table 1 and Text-Fig. 4 that the chromosomes do not show any abrupt increase in DNA along their length, and that the small increases do not build any regular pattern with a 3-micron interval. At the side of every autosome was placed the extinction value of the sex chromosome belonging to the same or an adjacent nucleus (Text-Fig. 4). Due to the presence of the reference beam, not all chromosomes may be measured within the same nucleus as they may be located over this beam. Thus, one sometimes needs to measure the sex chromosome of an adjacent nucleus. The sex chromosome shows a DNA content that is 3 to 4 times higher than the autosomal segments.

It has been previously ascertained by means of grain counts combined with spectrophotometry that there is good agreement between the number of silver grains and the DNA content of the chromosomes in this species. The number of silver grains per unit area present in the sex chromosome is 2 to 3 times higher than in the autosomes, and its DNA content is correspondingly 3 to 4 times higher than that of the autosomes (LIMA-DE-FARIA, 1959 b, Tables 1 and 2 and Text-Fig. 4). This means that a doubling of the grain number corresponds to at least a doubling or triplication of the DNA content of the segment studied. This relation applies equally for the present study since we are dealing with the same species and nuclei with a similar grain density, only studied at a later stage. The clusters of grains studied here at late pachytene may contain as many as 12 grains, whereas the adjacent sections have none or one. If the grain clusters were the result of a higher DNA content of the segment where they occur, the amount of this substance should be at least as high as in the sex chromosome, but it is not. No value in the autosome segments approaches that of the sex chromosome (Table 1 and Text-Fig. 4).

To find out, however, if the segments which exhibit the clusters actually contain more DNA or not, another series of measurements was performed. The chromosomes containing grain clusters distributed at re-



Text-Fig. 5. Graphic representation of extinction values of the same chromosome segments before (first horizontal column) and after (second horizontal column) removal of silver grains. Data from Table 2. ⁎=grain cluster. ×=part of a cluster or 1 or 2 silver grains.

gular intervals were measured (Table 2, horizontal column headed "silver grains present", and Text-Fig. 5). The high values over the silver grains have no significance from the DNA point of view. They represent the absorption of the silver, and serve only to mark the position of the clusters. After that the silver grains were removed and the same chromosomes relocated in the apparatus and measured again (Table 2 and Text-Fig. 5, second horizontal column for each chromosome).

An analysis of variance of the material shows (Table 3, regions I and III) that there is no statistical significant difference between the DNA amount of the regions that contained silver grains and the regions that were free of them, $P > 0.2$. The same P-value is obtained when one tests regions that had no grains with those that had clusters and 1 or 2 grains (Table 3, regions I and II). Table 3 also shows that the ferricyanide treatment used to remove the silver grains also removed a significant amount of DNA from the chromosomes under the conditions of our experiment, $P < 0.001$ (regions I and IV in Table 3). However, since the removal affected all chromosome segments in a similar way (Table 2), this general decrease does not influence the test between the segments that previously possessed or lacked clusters of grains.

DISCUSSION

FREESE (1958) discussed at length the organization of the DNA molecule in the chromosome. The main question brought forward was whether all the genetic information of a chromosome was contained in one long double helix or whether the chromosome contains a large number of smaller DNA molecules.

Although no direct evidence was available FREESE was led to consider a chromosome composed of a large number of smaller DNA molecules to be the most likely. He proposed a chromosome model where the DNA tape is constituted of a number of smaller units united in a one dimensional chromosomal thread.

Support for the choice of this model was drawn from the following sources.

(1) In all DNA preparations analyzed the molecular weight has been found to be about 10 million, and a DNA molecule with this average molecular weight can be only a very small fraction of the total DNA of a chromosome. Chromosomes of higher organisms have a DNA content which corresponds to a total length of over one meter (TAYLOR,

1957). The length of a DNA molecule is considered to be 5 microns (FREESE, 1958).

(2) The results of MESELSON and STAHL (1958) suggest that the DNA replication involves a rotation of the DNA in order to unwind the templates. In unwinding a very long DNA thread might easily break. Then, a thread that is composed of smaller units that can freely rotate during their duplication would be more in agreement with the properties of molecular systems. The experiments of LEVINTHAL and THOMAS (1957) indicate that the DNA of phage may not be a single large unit but may be made up of a number of smaller molecules, the largest one of which makes up about 40 per cent of the total DNA of the phage particle. The molecular weight of the total DNA content of a T-2 phage particle has been calculated to be approximately 110 million. Several lines of evidence suggest that this segment comprising 40 per cent of the total DNA is composed of still smaller units having molecular weights of the order of 12 to 15 million.

The results described in this paper have a bearing on this problem as well as on crossing-over. They reveal that the meiotic chromosome is composed of minor segments where DNA replication is not taking place simultaneously. In drawing conclusions from this phenomenon it is necessary to take into consideration the two following points.

First, the autoradiographic picture observed at late pachytene is the result of a phenomenon which occurred at interphase. At this last stage the chromosome is organized in a different way, probably is much more distended.

Secondly, the 3 micron distance is the actual distance between the groups of grains but not the actual length of the DNA thread. In *Melanoplus* chromosomes the chromomeres can be seen to be constituted of spirals. This is a phenomenon also observed in other organisms (RIS, 1957; EBERLE, 1957). Thus, the spiralized DNA thread which makes up the 3 micron segment must actually be longer. However, of significance from the genetic point of view is the length of the segment found at pachytene since this is the stage at which crossing-over is supposed to occur.

Crossing-over between genes may more easily take place at the sites in the DNA molecule where replication is initiated than elsewhere. This would be in agreement with the non-random distribution of crossing over known to occur in many species (MATHER, 1940).

The same may be true as regards mutation. Mutation in the form of breakage and reunion may likewise more easily occur at the points of

asynchronous replication. The cytological literature of the last ten years on the action of chemicals on chromosomes abounds in examples of non-random breakage along the chromosome body (KIHLMAN and LEVAN, 1951; MCLEISH, 1953, 1954; MOUTSCHEN-DAHMEN, 1958; and others).

Giant chromosomes of Diptera which differ from pachytene chromosomes in their particular type of organization also exhibit an asynchrony of DNA synthesis (PAVAN, 1958; SIRLIN, 1960). Certain bands and interbands appear labelled with tritiated thymidine whereas others do not. Particularly interesting is that protein synthesis in salivary gland chromosomes also exhibits a localized pattern. The uptake of methionine-^{35}S and leucine-^{3}H results in a discontinuous labelling of the chromosome (SIRLIN and KNIGHT, 1960). In both DNA and protein synthesis no regular occurrence of labelled segments has so far been detected. This is not surprising in view of the complex organization of the giant chromosomes.

SUMMARY

Autoradiographic stripping film was applied over Feulgen squashes and sections of spermatocytes of the grasshopper *Melanoplus differentialis* after injection of tritiated thymidine into the body cavity. Testes were fixed 9 to 12 days following the injection.

In chromosomes at late pachytene the labelling was found to occur in clusters of silver grains distributed along the entire length of the chromosome body at approximately 3 microns intervals. As synthesis progresses the chromosomes get heavily labelled, and finally the number of silver grains starts tapering off until synthesis ends.

Measurements of the amount of DNA along the chromosome were undertaken using a highly sensitive automatic recording microspectrophotometer with a photocell aperture corresponding to an area in the plane of the object equal to 0.7×0.7 microns. The chromosomes were divided into 1.3 micron segments and the absorption measured for each segment.

In chromosomes without silver grains there is no abrupt increase in DNA content along the chromosome and the small increases do not build any regular pattern with a 3 micron interval. In chromosomes where clusters of silver grains are present spectrophotometric measurements were made before and after the silver grains were removed. An analysis of variance of the extinction values shows that there is no

statistical significant difference between the DNA content of the regions that contained silver grains and the regions that were free of them. The occurrence of the grain clusters at 3-micron intervals is thus not correlated with a higher DNA content of the chromosome segments involved.

The initiation of the DNA synthesis does not take place simultaneously along the whole meiotic chromosome, but rather occurs at specific segments.

These results have a bearing on crossing-over since this phenomenon occurs at meiosis and is connected with chromosome replication. Crossing-over between genes may more easily take place at the sites where DNA replication is initiated than elsewhere.

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THE PATTERN OF DNA SYNTHESIS IN THE CHROMOSOMES OF MAN

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INTRODUCTION

THE main interest in the chromosomes of Man centered originally on their significance in connection with the cancer problem (LEVAN and BIESELE, 1958). Later the importance of the study of human chromosomes has extended to a new and unexpected field. In a relatively large number of cases clinical syndromes have been found to be associated with variations in chromosome number and chromosome rearrangements (reviews in BÖÖK and SANTESSON, 1960; NACHTSHEIM, 1960).

These two main lines of research have led to active study on the chromosome complement of Man (TJIO and LEVAN, 1956; TJIO and PUCK, 1958; CHU and GILES, 1959; LEVAN and HSU, 1959; PATAU, 1960; and the "Human Chromosomes Study Group", 1960). The chromosomes have now been numbered and assembled into several groups mostly according to size, but several obscure points remain. The X chromosome is at mitotic metaphase, difficult to distinguish from the other autosomes of similar length. The Y chromosome has also a size and a kinetochore location that makes it difficult to separate it from the smallest autosomes but it is more readily identifiable than the X. Furthermore, the two X chromosomes of the female seem to have different genetic properties (OHNO and MAKINO, 1961).

In the interphase nuclei of many tissues of the human body a darkly stained body is seen which has a different appearance in females from that in males. This makes it possible to identify with great accuracy the sex of an individual. The heterochromatic body has been attributed to the sex chromosomes of Man.

Autoradiography has shown that tritium labelled DNA precursors can help to distinguish between different chromosomes of the complement when these are heterochromatic (LIMA-DE-FARIA, 1959 a and b).

In the grasshopper *Melanoplus differentialis* the large single X chromosome, present in the males, synthesizes DNA at a different period of time from the autosomes, and this permits its easy identification in the nucleus. This X chromosome is positively heteropycnotic at prophase of meiosis.

Thymidine, a specific precursor of DNA, when labelled with tritium, and taken up by chromosomes, leads to autoradiographic images that allow the study of DNA synthesis in chromosome segments as short as 1 to 2 microns. Tritiated thymidine was added to tissue cultures of human skin from embryos and to cultures of leukocytes from peripheral blood, with a view to studying the pattern of DNA synthesis in human chromosomes.

MATERIAL AND METHODS

A preliminary series of experiments was carried out using tissue cultures of embryonic fibroblasts. The human embryos 2 to 4 months old were obtained from the Lund University Obstetric Clinic. The culture medium used was Parker 199 with 20 % inactivated human serum to which was added 100 gamma streptomycin per ml, and 100 units of penicillin per ml. The cultures were incubated at 37° C. Carrel flasks were used for the outgrowth of the fibroblasts. The cells were then trypsinized and transferred to cover slips for further growth. These were then placed in ordinary tubes, and incubated for 2 days. After that the cover slips were transferred to a culture medium to which tritiated thymidine was added in a concentration of 3 μc per ml. This material was used to find out the most suitable experimental treatment of the chromosomes. The colchicine concentration, the amount of hypotonic treatment, the most suitable staining procedure, and the exposure time, were tested in different ways. The stain used must be one which is both suitable for studying the structure of human chromosomes and at the same time is not affected by the autoradiographic procedure, especially the developing of the film.

Fibroblast cultures furnish a large number of cell divisions and very good metaphase plates. However, when autoradiography is used a much higher number of divisions is necessary because only a certain percentage of the cells gets labelled. The number of first class metaphase plates where every chromosome can be analysed needs to be correspondingly much higher. The study becomes still more difficult due to the fact that when making the karyotypes every chromosome needs

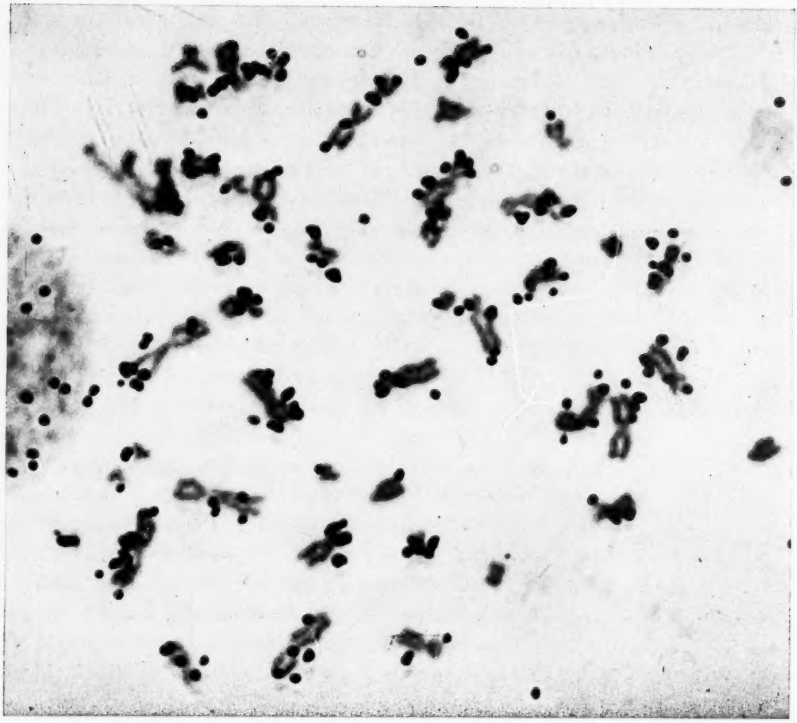

Fig. 1. Human leukocyte from peripheral blood. Metaphase with the 46 chromosomes of the normal complement labelled with tritiated thymidine. Note that not all chromosomes are labelled and that not all segments within a chromosome are replicating simultaneously. — ×2200.

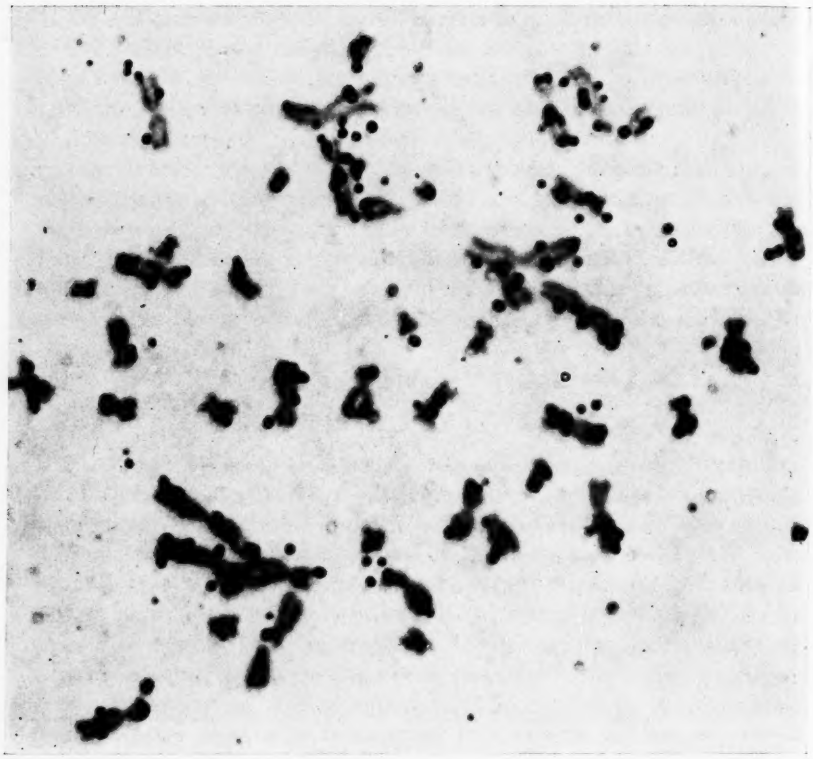

Fig. 2. Human leukocyte from peripheral blood treated with tritiated thymidine. The chromosomes show similar labelling as in Fig. 1. — ×2200.

to be analysed morphologically and at the same time is covered with silver grains. The karyotypes are already difficult to make when the chromosomes are studied without autoradiography due to the similarity of many chromosome pairs. When the silver grains of the emulsion cover the chromosomes one needs to reject cells where the labelling is too intense. In these cells the individual chromosomes cannot be recognized and although, the squashing may be excellent, a karyotype cannot easily be constructed. This also creates an additional difficulty in the printing of the karyotypes. When the chromosomes are photographed, there needs to be a contrast between silver grains and chromosome arms. As a result the chromosomes cannot be printed as dark as in ordinary photographs. When they are pasted side by side in the karyotype the outline of the photographic paper cannot easily vanish in the printing process, because the chromosomes are not as dark as in photographs where silver grains are absent.

After the main details of the technique were cleared up we found it necessary to shift to leukocyte cultures from peripheral blood because the number of divisions is much higher here (MOORHEAD *et al.*, 1960). The results described below are only from these cultures.

Blood of a human healthy male was used throughout all the experiments. It is a pleasure to acknowledge the help of our colleague Dr. B. KÄLLÉN with the preparation of the initial stages of the blood cultures. Twenty ml of venous blood were taken each time with the help of a syringe wet with heparin, and poured into two vials containing one drop of heparin and 2.5 ml of 6 % dextran. After that they were allowed to stand for 30—45 minutes. The supernatant was removed and centrifuged for 5 minutes at 2,000 r.p.m. After centrifugation the new supernatant was discarded and 5 cc. of medium were added. The sediment is mixed well with the medium and one drop is placed in a Bürker blood counting chamber to estimate the number of leukocytes. The cultures were diluted with additional medium to a concentration of 800,000 cells per ml. After that the culture was distributed by 10 small vials, each containing 1.5 ml of culture medium, and placed in an incubator at 37° C.

The medium used consists of 30 % human serum and 70 % Parker 199. To each 10 ml of this solution was added one drop of heparin, 0.2 ml of phytohemagglutinin and 5 ml tyrode solution. Five units of penicillin and five of streptomycin were used to each ml of serum.

Tritiated thymidine was added to the culture medium in a concentration of 3 μc per ml with a specific activity of 1.9 curie per mM. At

the end of the period of contact with the thymidine the culture tubes were centrifuged at 2,000 r.p.m. for 5 minutes. The culture medium with tritiated thymidine was poured out and the sediment containing the leukocytes washed twice thoroughly with Parker 199 free of tritiated thymidine. After this procedure, 1.5 ml of culture medium without thymidine was added to the sediment of leukocytes and these loosened from the bottom of the tube into the medium. This was found to be a quite efficient way of ridding the cells of tritiated thymidine. The culture was then transferred with a pipette to a new vial and placed in the incubator at 37° C for the period of time desired. After this period four drops of a colchicine solution 0.001 % were added. The cells remained in contact with the colchicine for 2 hours (at 37° C). Hypotonic treatment followed by adding two parts of distilled water to one part of the culture medium and letting the mixture remain at room temperature for 7 minutes. The material was then centrifuged as before and the leukocytes collected in the sediment. The supernatant was discarded and the cells were immediately fixed by adding acetic alcohol 1:3 to the sediment. The fixation time was 10 minutes. The fixative was then decanted and two drops of 45 % acetic acid added. With the help of a needle the cells were mixed with the acid solution and a small drop of this cell suspension placed over a subbed slide. The cover slip was placed in position and strong pressure applied. The preparations were at once inverted over dry ice, after which the cover slips were removed. The slides were then passed through absolute alcohol, 70 % alcohol, 35 % alcohol, for two minutes in each case and finally immersed in water.

The staining was performed by hydrolysing the cells in Normal HCl for 6½ minutes, passing them through water, and staining in fuchsin-sulphurous acid for one hour. Three rapid changes in SO_2 water followed, and the slides were finally immersed in water (three changes). Autoradiographic stripping film Kodak AR-10 was applied in the usual way. The film exposure time was two days.

The Feulgen staining of chromosomes is the most suitable when one works with autoradiography, since it withstands quite well the developing procedure of the film, but for human chromosomes the structural features were not as distinct as after the commonly used orcein staining. Orcein not being a specific stain of DNA is easily lost from the chromosomes during the autoradiographic procedure, and changes its hue with variations in pH. To attend to the advantages and drawbacks of both staining procedures a schedule was developed in which

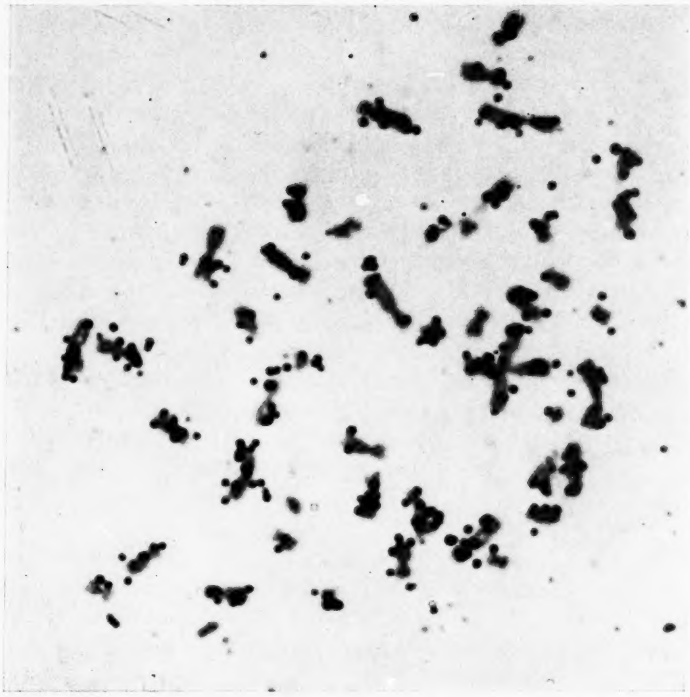

Fig. 3. Human leukocyte from peripheral blood treated with tritiated thymidine. The chromosomes show similar labelling as in Fig. 1. — ×2200.

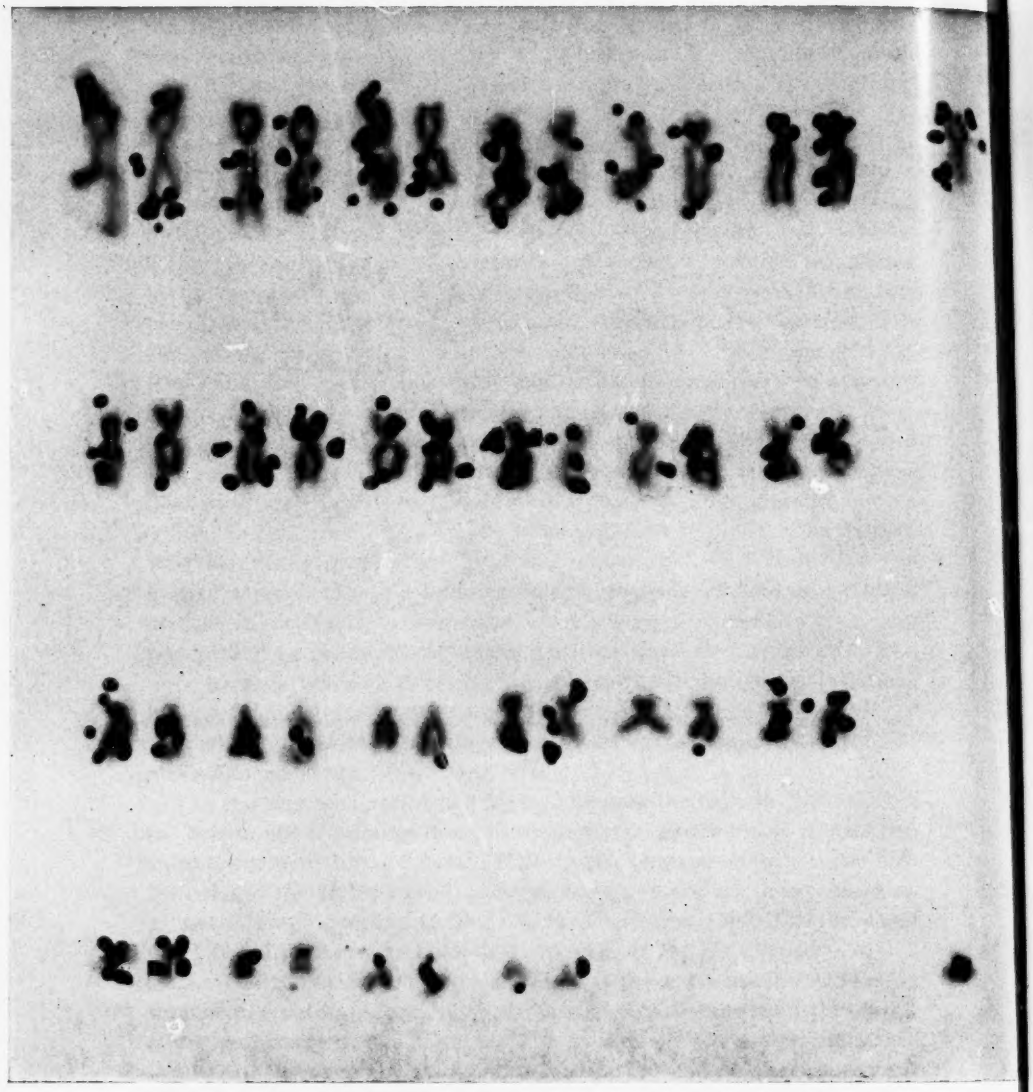

Fig. 4. Karyotype of human chromosomes from leukocyte exposed to tritiated thymidine. X and Y chromosomes are to the right of first and last horizontal column respectively. Due to the presence of the silver grains the contrast between the chromosomes and the background cannot be as sharp in autoradiographs as in the usual type of karyotype.

after the hypotonic treatment and centrifugation the cells were fixed in 60 % acetic acid for two minutes. The fixative was then poured out and two drops of 2 % orcein dissolved in 60 % acetic acid were pippeted over the leukocyte sediment. The acetic orcein acted for 5 minutes and a small drop of the cell emulsion was placed over a subbed slide. A cover slip was placed in position and strong pressure applied. The preparations were also inverted over dry ice after which the cover slips were removed. The slides were immersed in the same alcohol series as was used for the Feulgen staining and finally brought to water. These preparations stained with orcein were then restained with Feulgen by following the schedule described above. The cells in this case got a double staining which resisted the autoradiographic procedure better. The quality of the staining is uneven but in some cases may be as good as Feulgen, with the advantage that the chromosome structure is better preserved. The photographs shown in this paper are taken from preparations made after both staining procedures.

RESULTS

In peripheral venous blood leukocytes are not supposed to divide, but once they are placed in culture in contact with phytohemagglutinin, they start dividing and a maximum of divisions is known to occur by the third day of incubation.

A preliminary series of experiments was performed to find out the most suitable period of time for which the cells should be placed in contact with H^3-thymidine. In a first experiment, the cells, after 30 hours in culture, followed by 2 hours contact with tritiated thymidine, were allowed to be without tritium for 42 hours. Colchicine followed for 3 hours. In these preparations there are already labelled nuclei. This means that DNA synthesis occurs already 30 hours after the leukocytes have been placed in contact with phytohemagglutinin.

Two other experiments were performed in the same way, but the initial time in culture before tritium, was prolonged to 48 and 57 hours. The total time of the experiment was the same (77 hours). The number of silver grains per nucleus is lower in the cells of the first experiment than in the cultures that got the tritium at 48 h. and 57 h. after the start of the experiment. This indicates that the tracer was diluted in subsequent cell divisions, during the 42 hour period that followed exposure to tritium. The number of metaphases labelled is, as expected,

lower in these preparations than in those that were tritiated after 48 h. and 57 h. in culture.

Another series of experiments was performed to find out the suitable period during which the cells should be allowed to be incubated after having been in contact with tritium and the period during which they should be in contact with tritiated thymidine. For this purpose the cultures were incubated for 46 hours and subsequently exposed to tritiated thymidine for 90 min. and 120 min. The period of incubation between the end of exposure to tritium and fixation varied from 3 h. and 45 min. to 5 h. and 45 min. (Table 1). The number of metaphases labelled increased from 3 h. and 45 min. to 5 h. and 45 min., with a distinct maximum between 5 h. and 15 min. and 5 h. and 45 min. The percentage values in Tables 1—3 are based on counts made on more than one hundred cells. In a fifth experiment (Table 2) the tritium was applied 58 hours after incubation and the time of exposure to the tracer varied between 20 to 90 minutes, followed by a period without tritium of 3 h. and 30 min. to 9 hours. The time of exposure to H^3-thymidine was diminished successively to disclose any asynchrony in DNA replication that may be present in the chromosome complement. In this case the highest number of metaphases labelled was also found to increase from the 3 to 9 hours period, with a maximum after 4 h. and 30 min. (Table 2).

Since the time of exposure to the tracer varied in the two previous experiments, another one was performed in which all cultures were exposed to tritiated thymidine for only 20 minutes (Table 3). After 6 to 9 hours of incubation following exposure to tritium, the number of metaphases labelled was found to be 90 per cent or more. The number of metaphases with more than half of the chromosomes labelled was in every case much larger than the number of metaphases with less than half of the chromosomes labelled.

From these experiments the following characteristics are revealed. After 3 h. and 30 min. to 3 h. and 45 min. of contact with the tritium only 17 to 33 per cent of the metaphase plates are labelled. Between 4 h. and 30 min. and 5 h. and 45 min. after tritiated thymidine 31 to 90 per cent of the metaphase plates become labelled. This means that the interval between the end of DNA synthesis and metaphase varies between 4 to 5 hours under the conditions of our experiments. The two hour treatment with colchicine influences probably this value but we find it advantageous to obtain a perfect squash of the metaphase chromosomes. In this study colchicine cannot be avoided.

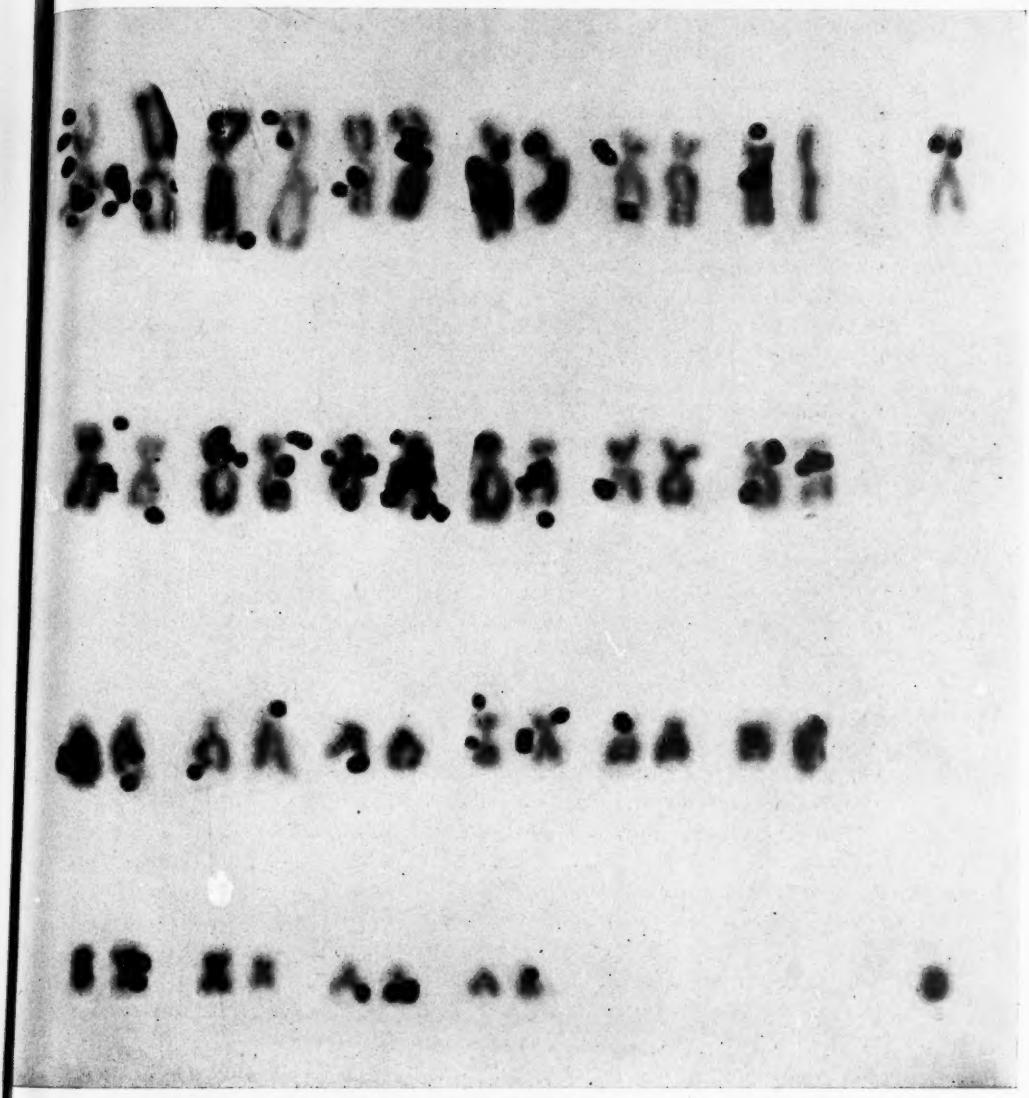

Fig. 5. Karyotype of human chromosomes from leukocyte treated with tritiated thymidine. For description see text. X and Y chromosomes located as in Fig. 4.

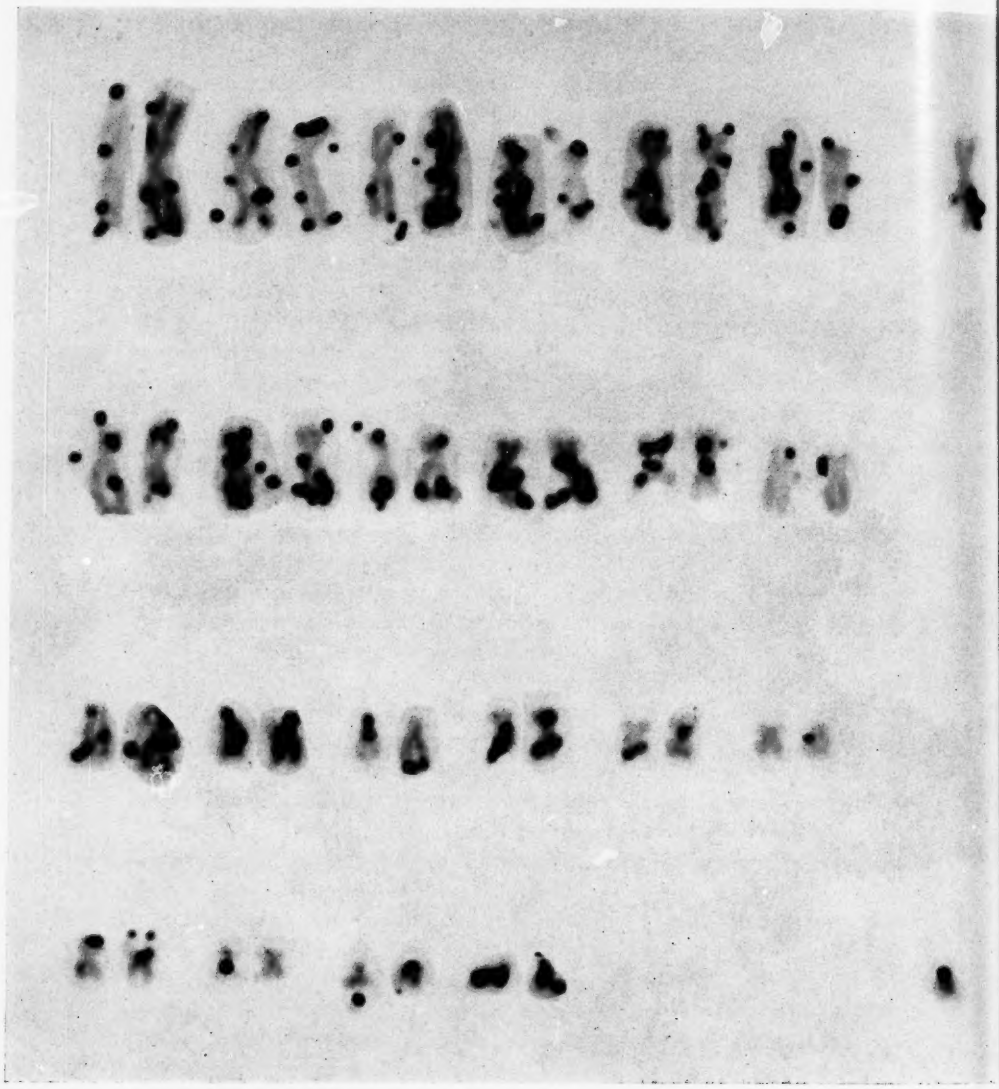

Fig. 6. Karyotype of human chromosomes from leukocyte treated with tritiated thymidine. For description see text. X and Y chromosomes located as in Fig. 4.

TABLE 1. *Schedule of treatment and percentages of labelled and unlabelled metaphase plates.*

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Number	In culture	In tritiated thymidine	Without tritiated thymidine	Colchicine	Total time after H^3 thymidine	Metaphases not labelled	Metaphases with less than half of chrom. lab.	Metaphases with more than half of chrom. lab.	Metaphases with labelled chromosomes
1	46 h.	90 m.	1 h. 45 m.	2 h.	3 h. 45 m.	70	23	7	30
2	46 h.	90 m.	2 h. 45 m.	2 h.	4 h. 45 m.	53	29	18	47
3	46 h.	120 m.	3 h. 15 m.	2 h.	5 h. 15 m.	34	23	43	66
4	46 h.	120 m.	3 h. 45 m.	2 h.	5 h. 45 m.	10	19	71	90

TABLE 2. *Schedule of treatment and percentages of labelled and unlabelled metaphase plates.*

Number	In culture	In tritiated thymidine	Without tritiated thymidine	Colchicine	Total time after H^3 thymidine	Metaphases not labelled	Metaphases with less than half of chrom. lab.	Metaphases with more than half of chrom. lab.	Metaphases with labelled chromosomes
1	58 h.	20 m.	1 h.	2 h. 30 m.	3 h. 30 m.	67	20	13	33
2	58 h.	45 m.	1 h.	2 h. 30 m.	3 h. 30 m.	83	10	7	17
3	58 h.	90 m.	2 h. 30 m.	2 h.	4 h. 30 m.	69	17	14	31
4	58 h.	75 m.	5 h.	2 h. 30 m.	7 h. 30 m.	24	26	50	76
5	58 h.	60 m.	7 h.	2 h.	9 h.	16	21	63	84

TABLE 3. *Schedule of treatment and percentages of labelled and unlabelled metaphase plates.*

Number	In culture	In tritiated thymidine	Without tritiated thymidine	Colchicine	Total time after H^3 thymidine	Metaphases not labelled	Metaphases with less than half of chrom. lab.	Metaphases with more than half of chrom. lab.	Metaphases with labelled chromosomes
1	60 h.	20 m.	2 h. 30 m.	2 h.	4 h. 30 m.	52	17	31	48
2	60 h.	20 m.	4 h.	2 h.	6 h.	10	24	66	90
3	60 h.	20 m.	5 h.	2 h.	7 h.	10	9	81	90
4	60 h.	20 m.	6 h.	2 h.	8 h.	5	13	82	95
5	60 h.	20 m.	7 h.	2 h.	9 h.	1	9	90	99

A significant characteristic of these results is the asynchrony in chromosome replication. It is found that at metaphase not all chromosomes are labelled, and that not all loci within a chromosome are labelled simultaneously (Figs. 1—3). The short exposure to tritiated thymidine (20 minutes) which is only a fraction of the total period of DNA synthesis, helps to put in evidence this phenomenon.

To study the asynchrony of DNA synthesis in human chromosomes karyotypes were made to compare in detail the 46 chromosomes of the complement (Figs. 4—6). From the analysis of the karyotypes the following results emerge. First, the largest chromosomes synthesize at a different period of time from some of the smallest and some of the medium-sized chromosomes.

Secondly, the two arms of the same chromosome do not always replicate simultaneously. This is better established in large chromosomes than in small ones, but the phenomenon seems to occur in all size categories.

Thirdly, there is a distinct difference in DNA synthesis between the two homologues of the same autosome pair. The presence of silver grains makes the construction of the karyotypes difficult but when cells with few silver grains are chosen the chromosomes can be grouped in pairs without difficulty. The difference in DNA synthesis among the homologous autosomes is particularly distinct in the large chromosomes. Its occurrence in chromosomes of medium and small size is more difficult to establish due to the asynchrony of the loci within the chromosomes, but it has been observed so often in these two size categories that it most probably also occurs in these chromosomes.

Fourthly, the X chromosome cannot be easily distinguished from the other chromosomes of the complement of similar size, but in the karyotypes made, the chromosome recognized as the X was found to synthesize DNA at a different period of time from some of the medium sized and small chromosomes. Furthermore, both arms of the X do not synthesize at the same time. In some karyotypes the short arm is seen labelled when the long arm is unlabelled, whereas in others the reverse is observed. There is also a period of DNA synthesis where both arms of the X chromosome are labelled simultaneously (Figs. 4—6).

The Y chromosome is usually not labelled when most chromosomes of the complement, including the X, are labelled. When the Y is labelled, it is normally out of step with some of the smaller chromosomes of the complement.

DISCUSSION

Although the pattern of replication is quite complex it is not much different from that of other organisms. That different chromosomes of the complement synthesize at different periods of time has been found in insects (LIMA-DE-FARIA, 1959 a and b) and in mammals (TAYLOR, 1960). That the sex chromosomes synthesize at a different period of time from the autosomes has also been found in the grasshopper *Melanoplus* (LIMA-DE-FARIA, 1959 b) and in the Chinese hamster (TAYLOR, 1960). The finding that the two arms of the X chromosome synthesize at different periods of time is also known to occur in the hamster. But the different time of synthesis among the homologous chromosomes of a pair, observed in the autosomes, has not been so far reported for another species. This increases strongly the asynchrony of the replication picture of the whole complement.

It is this extreme asynchrony that is rather striking. The pattern of DNA replication in human chromosomes is the most complex so far encountered. This is due to: (1) the high chromosome number, (2) the high asynchrony of the loci within a chromosome, and (3) the asynchrony among homologous chromosomes. This is an indication that the chromosomes of Man are strongly diversified and have large potentialities for differentiation.

It will be of special interest to investigate patients with leukemia and healthy individuals belonging to different geographical areas of the world to see whether they have different patterns of DNA replication.

Acknowledgement. — This investigation was supported by a research grant from the Swedish Natural Science Research Council to the senior author.

SUMMARY

Human chromosomes from leukocyte cultures of peripheral blood were labelled with tritiated thymidine.

DNA synthesis occurs already 30 hours after the beginning of the incubation period, but the maximum number of labelled divisions is seen after 60 hours.

The interval between the end of DNA synthesis and metaphase varies between 4 to 5 hours.

Not all loci in the 46 chromosomes of man are synthesizing simultaneously. The large chromosomes replicate at a different period of time from some of the smaller ones. The two homologues of most

chromosomes pairs also duplicate at different periods of time. The two arms of the chromosome recognized as the X do not replicate at the same time, and the Y chromosome is also out of step with some of the smaller chromosomes of the complement.

The pattern of DNA replication in Man is the most complex so far encountered. No nucleus has been found where all loci of the 46 chromosomes replicate simultaneously.

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BRIEF REPORT

ARNE LUNDQVIST: A rapid method for the analysis of incompatibilities in grasses.

(Received September 27th, 1961)

In the majority of self-incompatible angiosperms pollen tube growth or even the germination of the pollen grain is inhibited when pollen and pistil are incompatible. JOST (1907) and HAYMAN (1956) demonstrated that the grass pistil is admirably suited for a cytological check of this inhibition, since the feathery stigmas are easily spread out under a cover glass so that the pollen grains applied to them on the fine stigmatic branches can be distinctly seen in the microscope. With the cytoplasmic stain cotton-blue in lactophenol (WATKINS, 1925) HAYMAN showed that the compatible pollen grains become rapidly emptied into the pollen tube and stain but faintly, whereas the incompatible grains stain darkly since their cytoplasm is not all released into the pollen tube. It is obvious that the genetics of the incompatibility inter-relationships may be more rapidly and accurately analysed by this criterion than by the criterion of seed-setting.

Before the particular stigma can be pollinated and studied in the microscope, the floret also must be emasculated and dissected. Owing to the dense inflorescences it is a tedious and difficult job to make controlled pollinations in grasses. Small florets are easily damaged at emasculation, and without extensive thinning of the inflorescence contaminations may be difficult to avoid at pollination. An even germination of the pollen on the stigma is desirable, but this necessitates the pollen dust being evenly and not too densely applied to the stigmas. From these points of view it has proved extremely useful to combine emasculation and dissection and to pollinate the stigmas outside the floret.

To prevent drying, the dissected stigmas may be kept floating on bare tap water, and pollinated. However, many pollen grains burst upon contact with the water, and it is undesirable that the stigmas should be able to move about on the surface of the water. An agar plate is a sufficiently stable medium to provide the moisture required.

Whole pistils are dissected out from florets just ready for anthesis, and the ovary is carefully planted into the agar plate so that the stigmas are standing free from the medium. More than a hundred plants may be represented each with one pistil within the restricted area of a small petri dish. Pollen is dusted over this field of stigmas in a moment, and the petri dish is covered and kept in room temperature for 24—36 hours to allow germination and growth of the pollen. The petri dishes may afterwards be kept in a refrigerator until the cotton blue slides can be prepared.

Quite apart from its rapidity this method has several advantages. First, one can do with a very restricted supply of experimental material since on a single inflorescence the pistils may suffice for several petri dishes and the remaining florets be used as pollinators, the restricted area to be pollinated requiring but a minimum of pollen. Secondly, the range of experimental error becomes narrowed since in a particular pollination all the stigmas are dusted

over with the same cloud of pollen and the stigmas within the same petri dish are kept under uniform conditions. At the same time the amount of pollen applied to the stigmas is more readily controlled and contaminations by illegitimate pollen are avoided. Thirdly, the physiological conditions, specifically the approach to the optimal degree of humidity in the air, seem to be more easily controlled in the closed space of the petri dish, judging from the rate of emptied pollen in comparable pollinations made on pistils *in situ* (straws with panicles of *Festuca pratensis* kept in water) (Table 1). In fact there seems to be a more even germination of the pollen in the petri dish, with a higher rate of emptied pollen.

TABLE 1. *Proportion of emptied pollen grains in compatible sibmatings, in relation to method of pollination, species, and percentage of compatible pollen expected.*

Pollination	Species	Per cent emptied (compatible) pollen grains																					
		Expected	Observed 5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	Mean
In situ	1	50		1	1	2	9	17	21	17	17	12	2									39.8
	1	100							1	1	—	8	5	7	4	7	8	6	4	1		68.5
In petri dish	1	100						1	—	2	1	2	4	4	13	19	26	31	25	12	2		73.0
	2	50	1	2	2	1	8	8	13	15	14	10	4	2	—	1							40.9
	2	100									2	2	7	6	4	9	13	8	4	1			71.6
	3	50							1	1	3	10	1	1	3								48.5

Species: 1=*Festuca pratensis* (pollinations *in situ* made in 1959) (LUNDQVIST, 1961),
2=*Hordeum bulbosum*, and
3=*Secale cereale*.

The cross-fertilizing grass species investigated in Table 1 have all responded well to the method, as have also *Bromus inermis* (LUNDQVIST, unpublished) and the self-fertilizing barley (U. LUNDQVIST, personal communication). The only disadvantage so far observed is that burst pollen may obtain on the small stigmas of *Festuca pratensis*, and sometimes in this species filled pollen grains may fail to stain densely (LUNDQVIST, 1962).

The method has not been tried out in its details. It may turn out practicable to grow the pistils on any sufficiently moist and stable medium. The present materials have been grown on a medium containing 2 % agar, 10 % sucrose, and 100 ppm boric acid (the mixture suggested to me by Dr. J. L. BREWBAKER, USA). If the petri dish is covered while the medium is still hot, the vapor will condense on the cover and no additional moisture be necessary. Planted stigmas grow perceptibly on this medium and may be used for pollination tests

at least 12 hours after planting. Since it is difficult to avoid infections by fungi which exert a marked negative influence on the growth of the pollen, the best results were obtained when the stigmas were pollinated but a few hours after planting. As is the case when the stigmas are pollinated *in situ*, it is important that completely fresh pollen be dusted, immediately upon anthesis. On the other hand, emasculated florets can be preserved for a few days on the inflorescence before planting on the agar plate.

Since emasculation and dissection of the floret may be combined, the size of the floret will no longer be a really restricting factor in the study of incompatibilities in grasses. At the same time this method will provide the plant breeder in grasses with a simple and rapid method of controlling the presence of incompatibility relationships in a polycross system, or of contaminants in the breeding material.

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